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Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)

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SECTION 1: INTRODUCTION

The Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR or LT2 rule) requires public water systems (PWSs) that use surface water or ground water under the direct influence of surface water to monitor their source water (influent water prior to treatment) for *Cryptosporidium*, *E. coli*, and turbidity for a limited period [40 CFR part 141.701 (a)-(h)]. In support of the monitoring requirements specified by the rule, three documents have been developed to provide guidance to the affected PWSs and the laboratories that support them:

- *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)* (this document). This guidance manual for PWSs affected by the rule provides information on laboratory contracting, sample collection procedures, and data evaluation and interpretation advice.

- *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*. The goal of this manual is to provide *Cryptosporidium* and *E. coli* laboratories analyzing samples in support of the LT2 rule with guidance and detailed procedures for all aspects of microbial analyses under the rule to maximize data quality and consistency.

- *Users' Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) Data Collection System*. This manual provides PWSs and laboratories with instructions on the entry, review, and approval of electronic data using the LT2 Data Collection System, and for generating reports using the system.

All of these manuals are available at http://www.epa.gov/safewater/lt2/index.html. Responses to frequently asked questions (FAQs) on sampling, analysis, and data reporting questions for the LT2 rule also are available on this website.

This guidance document is provided to help implement the LT2 rule. This guidance document does not, however, substitute for the LT2 rule or the analytical methods approved for use under the rule. The material presented is intended solely for guidance and does not alter any regulatory or analytical method requirements not altered by the LT2 rule itself.

This manual provides guidance on the following aspects of the LT2 rule:

- **Section 1:** Overview of the rule's monitoring requirements and how the *Cryptosporidium* and *E. coli* data collected under the rule will be used
- **Section 2:** Guidance on submitting historical data ("grandfathering")
- **Section 3:** Understanding *Cryptosporidium* analyses
- **Section 4:** Understanding *E. coli* analyses
- **Section 5:** Establishing a *Cryptosporidium* laboratory contract
- **Section 6:** Guidance on collecting and shipping LT2 monitoring samples
- **Section 7:** Reviewing *Cryptosporidium* data
- **Section 8:** Reviewing *E. coli* data

1.1 Background

The LT2 rule is a National Primary Drinking Water Regulation that requires monitoring, reporting, and public notification requirements for all PWSs that use surface water sources. The rule requires additional treatment techniques for some systems, based on *Cryptosporidium* monitoring results (40 CFR part 141.720 - 141.721). The LT2 rule was developed to improve control of microbial pathogens, including specifically the protozoan *Cryptosporidium*, in drinking water and to address risk trade-offs with disinfection byproducts.

The LT2 rule provides for increased protection against microbial pathogens in public water systems that use surface water sources. The rule focuses on *Cryptosporidium*, a protozoan pathogen that is widespread in surface waters. EPA is particularly concerned about *Cryptosporidium* because it is highly resistant to inactivation by standard disinfection practices. Ingestion of *Cryptosporidium* oocysts can cause acute gastrointestinal illness, and symptoms in sensitive subpopulations may be severe, including risk of mortality. *Cryptosporidium* has been identified as the pathogenic agent in a number of waterborne disease outbreaks.

EPA convened a Federal Advisory Committee to develop recommendations for both the Stage 2 Disinfectants and Disinfection Byproducts Rule and the LT2 rule. As recommended by the Federal Advisory Committee, the LT2 rule requires public water systems that use surface water or ground water under the direct influence of surface water to monitor their source water (influent water prior to treatment plant) for *Cryptosporidium*, *E. coli,* and turbidity [40 CFR part 141.701 (a)-(h)]. These data would be used to determine whether additional treatment is needed at PWSs and to assess whether a relationship could be established between the *Cryptosporidium* and *E. coli* levels in source water.

1.2 Large System Requirements

Large systems affected by the LT2 rule include both filtered and unfiltered systems.

- A large, filtered system in the LT2 rule is a system that:
  - Uses surface water or ground water under the direct influence of surface water
  - Serves at least 10,000 people
  - Provides filtration or is unfiltered, but required to install filtration because the system no longer meets all filtration avoidance criteria

  Large, filtered systems are required to conduct initial source water monitoring that includes *Cryptosporidium*, *E. coli*, and turbidity sampling [40 CFR part 141.701 (b)].

- A large, unfiltered system in the LT2 rule is a system that:
  - Uses surface water or ground water under the direct influence of surface water
  - Serves at least 10,000 people
  - Does not currently provide filtration and meets all filtration avoidance criteria

  Large unfiltered systems are required to conduct initial source water monitoring that includes *Cryptosporidium* sampling only [40 CFR part 141.701 (d)].

All of the *Cryptosporidium* sampling requirements and guidance discussed in this document apply equally to both filtered and unfiltered systems and both filtered and unfiltered systems that serve at least 10,000

people are referred to as large systems in this document. However, the *E. coli* and turbidity guidance in this document does not apply to large unfiltered systems.

The steps required for LT2 rule compliance for large systems, and the schedule for these steps, are summarized in **Table 1-1**.

Table 1-1. Timeline for Large Systems Regulated under the LT2 Rule

Event	Schedule	Duration
Establish contract with a *Cryptosporidium* laboratory pending approval under EPA's Lab QA Program (Section 2.4.1, below)	As soon as possible	N/A - single event
Verify that your utility laboratory is certified under the drinking water laboratory certification program to perform the technique you plan to use for performing *E. coli* analyses under LT2[a]	As soon as possible	N/A - single event
Submit grandfathered *Cryptosporidium* data package	Within 2 months of rule promulgation[b] Within 8 months of rule promulgation[c]	N/A - single event
Work with your *Cryptosporidium* laboratory to establish a mutually acceptable sampling schedule	As soon as possible after establishing contract	N/A - single event
Submit sampling schedule through the LT2 Data Collection System	Within 3 months of rule promulgation	N/A - single event
Collect monitoring samples[d]	Beginning 6 months after rule promulgation	At least once per month for 2 years[e]
Submit monitoring results[d]	No later than 10 days after the end of the first month following the month that the sample was collected (approximately 40 to 70 days after sample collection, depending on when during the month the sample is collected)	At least once per month for 2 years[e]

[a] Not applicable to large, *unfiltered* systems because these systems are not required to monitor for *E. coli* or turbidity
[b] PWSs with at least 2 years of grandfathered data at the time of LT2 rule promulgation and that intend to use these data in lieu of monitoring under the LT2 rule
[c] PWSs with fewer than 2 years of grandfathered data at the time of LT2 rule promulgation, or that have at least 2 years of grandfathered data but intend to conduct monitoring under the LT2 rule
[d] PWSs may be eligible to use historical (grandfathered) data in lieu of these requirements if certain quality assurance and quality control criteria are met (see Section 2)
[e] PWSs monitoring for *Cryptosporidium* may collect more than one sample per month if sampling is evenly spaced over the monitoring period
N/A = Not applicable

1.3 Small System Requirements

A small system in the LT2 rule is a system that:

- Uses surface water or ground water under the direct influence of surface water
- Serves fewer than 10,000 people
- Provides filtration or is unfiltered but required to install filtration because the system no longer meets all filtration avoidance criteria

- Does not currently provide filtration and meets all filtration avoidance criteria

These systems are required to conduct initial source water monitoring for *E. coli* as an indicator of *Cryptosporidium* and, for those systems exceeding *E. coli* trigger levels, *Cryptosporidium* monitoring [40 CFR part 141.701 (c)].

The steps required for LT2 rule compliance for small systems, and the schedule for these steps, are summarized in **Table 1-2**.

Table 1-2. Timeline for Small Systems Regulated under the LT2 Rule

Event	Schedule	Duration
Verify that your utility laboratory is certified under the drinking water laboratory certification program to perform the technique you plan to use for perform *E. coli* analyses under LT2	Prior to rule promulgation	N/A - single event
Submit sampling schedule through the LT2 Data Collection System	Within 27 months of rule promulgation	N/A - single event
Collect *E. coli* samples	Beginning 30 months (2.5 years) after rule promulgation	1 year (2 samples per month)
Submit *E. coli* monitoring results	No later than 10 days after the end of the first month following the month that the sample was collected (approximately 40 to 70 days after sample collection, depending on when during the month the sample is collected)	At least once per month for 1 year
Possible additional monitoring requirement for *Cryptosporidium* if small systems exceed *E. coli* trigger levels[a]		
Establish contract with a *Cryptosporidium* laboratory pending approval under EPA's Lab QA Program (Section 2.4.1, below)	As soon as possible after you are notified that your plant has exceeded the *E. coli* trigger levels	N/A - single event
Submit sampling schedule through the LT2 Data Collection System	Within 45 months of rule promulgation	N/A - single event
Work with your *Cryptosporidium* laboratory to establish a mutually acceptable sampling schedule	Within 2 months of rule promulgation	N/A - single event
Collect *Cryptosporidium* samples	48 months (4 years) after promulgation	1 year (2 samples per month)[b]
Submit *Cryptosporidium* monitoring results	No later than 10 days after the end of the first month following the month that the sample was collected (approximately 40 to 70 days after sample collection, depending on when during the month the sample is collected)	At least once per month for 1 year

[a] Small systems may be required to monitor for *Cryptosporidium* for 1 year, beginning 6 months after completion of *E. coli* monitoring; *Cryptosporidium* monitoring required if the *E. coli* annual mean concentrations exceed 10 *E. coli*/100 mL for systems using lakes/reservoirs or exceed 50 *E. coli*/100 mL for systems using flowing streams

[b] PWSs monitoring for *Cryptosporidium* may collect more than two samples per month if sampling is evenly spaced over the monitoring period

N/A = Not applicable

Details on the use of the *Cryptosporidium* and *E. coli* data collected under the LT2 rule are provided in Sections 1.4 and 1.5.

1.4 Use of *Cryptosporidium* Data

Two types of *Cryptosporidium* data are collected under the LT2 rule: *Cryptosporidium* occurrence data from the analysis of monitoring samples, and method performance data from the analysis of matrix spike (MS) samples. The use of occurrence data from monitoring samples is discussed in Section 1.4.1; the use of method performance data from MS samples is discussed in Section 1.4.2.

1.4.1 *Cryptosporidium* Monitoring Sample Data

The concentration of *Cryptosporidium* oocysts in source water samples analyzed during the LT2 rule will be used to calculate a mean *Cryptosporidium* concentration for a PWS and classify the PWSs into a treatment requirements "bin" (40 CFR part 141.709). These bin classifications are provided in **Table 1-3.** The treatment bin classification established for each PWSs will be used to determine whether additional treatment is needed. PWSs in Bin 1 are not required to implement additional treatment. PWSs in Bins 2 - 4 will be required to implement increasing levels of treatment and source water protection to address their greater risk for high *Cryptosporidium* source water concentrations.

Table 1-3. Bin Classifications

***Cryptosporidium* Bin Concentration**	**Bin Classification**
Cryptosporidium < 0.075 oocysts/L	Bin 1
0.075 oocysts/L ≤ *Cryptosporidium* < 1.0 oocyst/L	Bin 2
1.0 oocyst/L ≤ *Cryptosporidium* < 3.0 oocysts/L	Bin 3
Cryptosporidium ≥ 3.0 oocysts/L	Bin 4

1.4.1.1 Calculating Bin Classifications

The method used to average individual sample concentrations to determine a PWS's bin classification depends on the number of samples collected and the length of the sampling period.
For a PWS serving at least 10,000 people, bin classification would be based on the following:

- For PWSs that collect at least 48 samples during the required monitoring period, the *Cryptosporidium* bin calculation is equal to the arithmetic mean of all sample concentrations
- For PWSs that collect at least 24 samples, but not more than 47 samples, during the required monitoring period, the *Cryptosporidium* bin calculation is equal to the highest arithmetic mean of all sample concentrations in any 12 consecutive months in the monitoring period

For PWS serving fewer than 10,000 people, and that monitor for *Cryptosporidium* for 1 year, bin classification would be based on the simple arithmetic mean of all sample concentrations.

In all cases, the bin concentration is calculated using individual sample concentrations. These concentrations are calculated as "number of oocysts detected / volume (in L) analyzed." Individual sample concentrations are not calculated as "oocysts detected / 10 L," nor are bin concentrations calculated as the "sum of the oocysts detected / the sum of the volumes analyzed." As a result, each sample has an equal weight on the final bin concentration. In cases where no oocysts are detected, the number of oocysts used to calculate the sample concentration is "0."

1.4.1.2 Number of Oocysts Detected Versus Bin Classification

To better understand the relationship between the absolute number of oocysts detected in your samples and the resulting bin classification, several crosswalks are provided below. **Table 1-4** applies to large plants conducting monthly monitoring over 2 years. This table provides a crosswalk between the sum of the oocysts detected in 10- and 50-L samples during the highest 12-month period and the corresponding bin classification.

Table 1-4. Effect of the Number of Oocysts on Bin Classification Based on Mean of 12 Samples

Sum of oocysts found in 12, 10-L samples[a]	Sum of oocysts found in 12, 50-L samples[b]	Corresponding range of mean *Cryptosporidium* concentrations		Corresponding bin classification
		From	To	
0 - 8 oocysts	0 - 44 oocysts	< 0.075 oocysts/L		1
9 - 125 oocysts	45 - 629 oocysts	0.075 oocysts/L	< 1.0 oocyst/L	2
126 - 365 oocysts	630 - 1829 oocysts	1.0 oocyst/L	< 3.0 oocysts/L	3
366 or more oocysts	1830 or more oocysts	≥ 3.0 oocysts/L		4

[a] Representing the highest 12-month mean; assumes that 10-L samples are analyzed for each event
[b] Representing the highest 12-month mean; assumes that 50-L samples are analyzed for each event

Table 1-5 applies to large plants conducting semimonthly monitoring over 2 years. This table provides a crosswalk between the sum of the number of oocysts detected in samples during the entire 2-year monitoring period and the corresponding bin classification. Again, because this crosswalk is based on analysis of exactly 10 L or 50 L for all samples, it may not apply to all plants that monitor for *Cryptosporidium* on a semimonthly basis, but it helps put into perspective the impact that one high sample result may have on bin classification.

Table 1-5. Effect of the Number of Oocysts on Bin Classification Based on Mean of 48 Samples

Sum of oocysts found in 48, 10-L samples[a]	Sum of oocysts found in 48, 50-L samples[b]	Corresponding range of mean *Cryptosporidium* concentrations		Corresponding bin classification
		From	To	
0 - 35	0 - 179 oocysts	< 0.075 oocysts/L		1
36 - 503	180 - 2519 oocyst	0.075 oocysts/L	< 1.0 oocyst/L	2
504 - 1463	2520 - 7319 oocysts	1.0 oocyst/L	< 3.0 oocysts/L	3
1464 or more	7320 or more oocysts	≥3.0 oocysts/L		4

[a] Assumes that 10-L samples are analyzed for each event
[b] Assumes that 50-L samples are analyzed for each event

Systems may analyze larger volumes than 10 L, and larger volumes analyzed should increase analytical sensitivity (detection limit), provided method performance is acceptable. Because these tables are based on analysis of exactly 10 L or exactly 50 L for all samples, it may not apply to all plants that monitor monthly for *Cryptosporidium*, but it helps put into perspective the impact that one high sample result may have on bin classification. In addition, filtering higher volumes may not result in the same high volume analyzed if the source is turbid and the PWS analyzes only a portion of the concentrated sample. The calculations used to determine the volume analyzed if less than the entire sample volume is analyzed are discussed in Section 7.5.3.

1.4.2 *Cryptosporidium* Matrix Spike Data

During LT2 rule *Cryptosporidium* monitoring, PWSs are required to collect one matrix spike (MS) sample for every 20 monitoring samples from their source water, per the requirements in EPA Methods

1622/1623 (Section 9.1.8). A description of MS samples is provided in Section 3.2.7 of this document. For large systems that perform monthly monitoring for 2 years and collect 24 monitoring samples and for small systems that are triggered into monitoring for 1 year and collect 24 monitoring samples, two MS samples will be analyzed. For large systems that perform semimonthly or more frequent monitoring for 2 years and collect 48 or more samples, a minimum of three MS samples will be analyzed.

Although MS sample results will not be used to adjust *Cryptosporidium* recoveries at any individual source water, the results will be used collectively to assess overall recovery and variability for EPA Method 1622/1623 in source water. The descriptive statistics of the MS sample results will be compared to the performance of the methods during the Information Collection Rule Supplemental Surveys to verify the assumptions on method performance upon which the LT2 rule is based.

When considering the method performance that could be achieved for analysis of *Cryptosporidium* under the LT2 rule, the Federal Advisory Committee (FACA) evaluated the results of EPA Methods 1622/1623 in the ICRSS, which involved 87 PWSs sampling twice per month over 1 year for *Cryptosporidium* and other parameters. During the ICRSS, the mean *Cryptosporidium* recovery and mean relative standard deviation of the MS samples were 43% and 49%, respectively (Reference 9.1).

1.5 Use of *E. coli* Data

E. coli data are being collected by large systems during LT2 rule monitoring to assess whether a relationship can be established between the *Cryptosporidium* and *E. coli* levels in source water and a microbial index developed to establish trigger levels for *E. coli* that would indicate high *Cryptosporidium* concentrations in a source water. If a relationship can be established, small systems initially will be permitted to monitor for *E. coli*, rather than conducting more expensive *Cryptosporidium* analyses. Only those systems with *E. coli* levels above the trigger level established in the microbial index would then be required to monitor for *Cryptosporidium* to determine bin placement (40 CFR part 141.702).

A preliminary index was developed during development of the FACA agreement using data from the Information Collection Rule (ICR) and ICRSS (Reference 9.2). These data were evaluated for parameters that could indicate the likelihood that a source water mean *Cryptosporidium* level would be above the Bin 2 threshold concentration of 0.075 oocysts/L. Fecal coliforms, total coliforms, *E. coli,* viruses (ICR only), and turbidity were assessed for development of the microbial index. Data analyses placed greater emphasis on *E. coli* and fecal coliforms because of the direct relationship between these parameters and fecal contamination.

E. coli was determined to provide the best performance as a *Cryptosporidium* indicator with the available data. Based on the data from the ICR and ICRSS, the preliminary *E. coli* trigger levels were set at a mean of 10 *E. coli*/100 mL for reservoir/lake-type source waters and 50 *E. coli*/100 mL for flowing stream–type source waters.

These levels may potentially be revised based on the much larger, more reliable *Cryptosporidium* and *E. coli* data set collected through LT2 rule monitoring.

SECTION 2: GRANDFATHERING *CRYPTOSPORIDIUM* DATA

"Grandfathered" *Cryptosporidium* data are results generated before monitoring under the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR or LT2 rule) starts and that a public water system (PWS) intends to use in determining its bin classification (Section 1.4.1) under the rule. Grandfathered data may be used in lieu of, or in addition to, results generated during LT2 rule implementation (40 CFR part 141.708). This section of the manual is designed to assist PWSs in producing grandfathered data that should be equivalent to the data collected during LT2 rule implementation and, therefore, eligible for use in bin classification. The final LT2 rule will establish requirements for reporting and acceptance of grandfathered monitoring results.

2.1 General Guidelines for Generating *Cryptosporidium* Data for Grandfathering

A PWS's grandfathered *Cryptosporidium* data package should meet the following general conditions (40 CFR part 141.708):

- Samples were collected from the appropriate location(s)
- Samples were representative of a plant's source water(s) and the source water(s) have not changed
- Samples were collected no less frequently than each calendar month on a regular schedule, beginning no earlier than January 1999 (when EPA Method 1622 was first released as an interlaboratory-validated method)
- Samples were collected in equal intervals of time over the entire collection period (e.g., weekly, twice-per-month, monthly)
- The data set includes all source water *Cryptosporidium* monitoring results generated during the grandfathered data monitoring period (see details below—data from monitoring not directed towards LT2 rule binning will not be a component of the binning data set)
- Sample volumes of at least 10 L were analyzed or, in cases where 10 L were not analyzed, at least 2 mL of packed pellet volume were analyzed (additional details below)
- The data were generated using the validated versions of EPA Methods 1622 or 1623
- The data are fully compliant with the QA/QC criteria specified in the version of Method 1622 or Method 1623 used to generate the data, including analysis of matrix spike (MS) samples at a frequency of at least 5% (1 MS sample for every 20 monitoring samples)

The following sections discuss these recommendations in more detail.

2.1.1 Sample Collection Location

The sample collection location requirements are the same for LT2 rule monitoring and for grandfathered data and are discussed in Section 6.2. If the PWS does not collect samples as recommended in Section 6.2, the data may not be acceptable for grandfathering.

2.1.2 Sample Collection Schedule

During LT2 rule monitoring, PWSs will be required to collect samples at least monthly and in accordance with a schedule established by the PWS prior to initiation of monitoring (40 CFR part 141.703). PWSs may collect samples more frequently (e.g., twice-per-month, weekly), provided the same frequency is maintained throughout the monitoring period [40 CFR part 141.701 (e)]. Sampling for grandfathered data should follow these same criteria.

EPA recommends that, prior to initiation of grandfathered monitoring, PWSs develop a schedule listing the calendar date on which each *Cryptosporidium* sample will be collected and include this schedule when submitting the grandfathered data package to EPA. PWSs that have begun grandfathered monitoring without establishing a sampling schedule should develop a schedule for the collection of remaining samples. PWSs should collect samples within 2 days before or after the dates indicated in their sampling schedules. Exceptions to the sampling schedule are noted as follows:

- If extreme conditions or situations exist that may pose danger to the sampler, or which are unforeseen or cannot be avoided and which cause the system to be unable to sample in the required time frame, the PWS should sample as close to the scheduled date as feasible and submit an explanation for the alternative sampling date with the analytical results.

- PWSs that are unable to report a valid *Cryptosporidium* analytical result for a scheduled sampling date due to failure to comply with the analytical method quality control standards (e.g., sample is lost or contaminated; laboratory exceeds an analytical method holding time) should collect a replacement sample within 14 days of being notified by the laboratory that a result cannot be reported for that date. PWSs should submit an explanation for the replacement sample with the analytical results.

Alternative sample collection dates should be timed so as not to coincide with another scheduled *Cryptosporidium* sample collection date. Documentation of alternate sample collection, including the reason, should be provided with the grandfathered data package.

Water treatment plants that use surface water or ground water under the direct influence (GWUDI), but are operated only seasonally (e.g., during times of high-water demand) should monitor at least monthly during the period when the plant is in operation.

The Federal Advisory Committee Agreement in Principle (Agreement) for the LT2 rule recommends that if PWSs collect a total of at least 48 samples (regardless of whether all of the samples were collected before LT2 rule promulgation or some were collected before and some after rule promulgation), the *Cryptosporidium* bin concentration will be equal to the arithmetic mean of all sample concentrations [40 CFR part 141.709 (b)(1)]. For PWSs that collect a total of at least 24 samples, but not more than 47 samples, the *Cryptosporidium* bin concentration will be equal to the highest arithmetic mean of all sample concentrations in any 12 consecutive months during which *Cryptosporidium* samples were collected [40 CFR part 141.709 (b)(2)].

2.1.3 *Cryptosporidium* Analytical Methods for Grandfathered Data

Methods 1622 or 1623 should be used for *Cryptosporidium* analyses for the LT2 rule [40 CFR part 141.708 (b)(1)]. The following are EPA-validated versions of Methods 1622 and 1623 acceptable for monitoring for *Cryptosporidium* before LT2 rule implementation:

- *Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA*. U.S. Environmental Protection Agency, Office of Water. 2001. EPA-821-R-01-025

- *Method 1622: Cryptosporidium in Water by Filtration/IMS/FA*. U.S. Environmental Protection Agency, Office of Water. 2001. EPA-821-R-01-026

- *Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA*. U.S. Environmental Protection Agency, Office of Water. 1999. EPA-821-R-99-006 *(**Note:** The 2001 version of the method should be used to generate data after January 1, 2002.)*
- *Method 1622: Cryptosporidium in Water by Filtration/IMS/FA*. U.S. Environmental Protection Agency, Office of Water. 1999. EPA-821-R-99-001 *(**Note:** The 2001 version of the method should be used to generate data after January 1, 2002.)*

The procedures in EPA Method 1622/1623 are performance-based, and allow for modifications. The 2001 versions of EPA Method 1622/1623 also approve for nationwide use modified versions of the methods using the following components:

- Whatman Nuclepore CrypTest® filter
- IDEXX Filta-Max™ filter
- Waterborne Aqua-Glo™ G/C Direct FL antibody stain
- Waterborne Crypt-a-Glo™ and Giardi-a-Glo™ antibody stains

Since release of the 2001 versions of Methods 1622/1623, EPA also has approved a modified version of the methods using the Pall Gelman Envirochek™ HV filter and has approved the use of irradiated, flow cytometer–sorted spiking suspensions for routine QC sample spiking.

Laboratories that analyze *Cryptosporidium* samples using other modified procedures, as allowed under the performance criteria of Methods 1622/1623, should be approved to use the modified procedures under the Lab QA Program discussed in Section 2.1.4, below, and in detail in the *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*.

Other notable differences between the 1999 and 2001 versions of EPA Method 1622/1623 include the following:

- Clarified sample acceptance criteria
- Modified capsule filter elution procedure
- Modified concentrate aspiration procedure
- Modified IMS acid dissociation procedure
- Updated QC acceptance criteria for initial precision and recovery (IPR) and ongoing precision and recovery (OPR) tests
- Addition of a troubleshooting section for QC failures
- Modified holding times
- Inclusion of flow cytometry–sorted spiking suspensions (required for spiked samples analyzed during LT2 monitoring)

2.1.3.1 Minimum Sample Volume and Subsampling Analysis

The requirements for sample volume analyses are the same for LT2 rule monitoring and for grandfathered data [40 CFR part 141.708 (b)(5)]. These requirements are discussed in Section 6.1 of this manual.

2.1.3.2 Analysis of Matrix Spike Samples

The requirements for analysis of matrix spike (MS) samples are the same for LT2 rule monitoring and for grandfathered data [40 CFR part 141.708 (e)]. These requirements, and guidance on MS sample collection, are discussed in Section 6.4.2 of this manual.

2.1.4 *Cryptosporidium* Laboratories for Grandfathered Data

EPA has established the Laboratory Quality Assurance Evaluation Program for the Analysis of *Cryptosporidium* in Water (Lab QA Program) to approve laboratories to perform *Cryptosporidium* analyses under the LT2 rule (see http://www.epa.gov/safewater/lt2/index.html). EPA recognizes that some PWSs could begin generating grandfathered *Cryptosporidium* data prior to when the Lab QA Program is fully implemented (e.g., before EPA is able to evaluate all laboratories that will participate in the program). Consequently, PWSs should ensure that their grandfathered *Cryptosporidium* samples are analyzed by laboratories that will be evaluated under the Lab QA Program before the data are submitted to EPA. Note that PWSs will not submit grandfathered data packages until after the LT2 rule is final, currently scheduled for mid- or late 2004. Samples analyzed by laboratories that do not meet the criteria for approval under the LT2 rule may not be accepted for grandfathering.

Laboratories should also participate in the EPA Protozoa PT Program. EPA does not expect there to be restrictions on the number of laboratories involved in the generation of a PWS's grandfathered data.

2.1.5 *E. coli* and Turbidity Measurements

The Agreement would not exclude the use of previously collected *Cryptosporidium* data if *E. coli* and turbidity samples are not collected. However, the Agreement recommends that PWSs serving at least 10,000 people should collect *E. coli* and turbidity samples along with *Cryptosporidium* samples when monitoring under the LT2 rule. EPA recommends that PWSs conducting early (i.e., grandfathered) monitoring collect and analyze *E. coli* samples with each *Cryptosporidium* sample and measure turbidity during each sampling event.

2.2 Reporting Grandfathered Data

The final LT2 rule will establish reporting requirements for grandfathered data. The following recommendations are intended to give PWSs an indication of potential reporting requirements for consideration when establishing their grandfathered data monitoring programs.

For consideration of grandfathered data, PWSs should submit to EPA a complete data package as described below.

2.2.1 Data Package Contents

The grandfathered data package should include the following:

1. A signed cover letter from the PWS certifying that the data represent the plant's current source water and that all source water *Cryptosporidium* monitoring results collected during the LT2 rule monitoring period (defined below) are included in the package
2. Sample collection schedule established before beginning monitoring
3. Where applicable, documentation addressing the dates and reason(s) for re-sampling, as well as the use of presedimentation, off-stream storage, or bank filtration during monitoring
4. A list of the field and MS samples submitted in the data package (see Section 2.2.1.1, below, for details), identified by sample ID and collection date
5. Sample results for all field and MS samples (see Section 2.2.1.2, below, for details) and
6. Documentation that all method-required quality control requirements were acceptable for every field and MS sample submitted with the package (see Section 2.2.1.3, below, for details).

2.2.1.1 Sample Results to be Reported

PWSs that conduct monitoring for grandfathering should submit results for all source water *Cryptosporidium* samples analyzed during the LT2 rule monitoring period, as defined below (40 CFR part 141.707). This will include all samples that were:

- Collected from the sampling location used for LT2 rule monitoring,
- Not spiked, and
- Analyzed using the laboratory's routine process for Method 1622/1623 analyses, including analytical technique and QA/QC.

EPA plans that the LT2 rule monitoring period for a specific PWS will begin with the collection of the first sample submitted for LT2 rule binning and end with the collection of the final sample submitted for LT2 rule binning (as long as a minimum of 2 years of acceptable data have been submitted). With the use of grandfathered data, the final sample may be collected before the end of the LT2 rule implementation schedule. Sample results generated after the last sample result in the PWS's data package would be considered outside the PWS's LT2 rule monitoring period and would not need to be submitted to EPA for LT2 rule binning purposes. However, these results may be subject to reporting requirements under other federal or State regulations.

2.2.1.2 Data Elements to be Reported for Each Sample Result

The following data elements, at a minimum, must be submitted for each *Cryptosporidium* monitoring sample and MS sample [40 CFR part 141.708 (d)]:

- PWS ID
- Facility ID
- Sample collection point
- Sample collection date
- Sample type (field or MS)
- Sample volume filtered (L), to nearest ¼ L
- Number of oocysts counted
- For samples in which less than 10 L is filtered or less than 100% of the sample volume is examined, PWSs should also report the number of filters used and the packed pellet volume.
- For samples in which less than 100% of sample volume is examined, PWSs should also report the volume of resuspended concentrate and volume of this resuspension processed through immunomagnetic separation.
- For matrix spike samples, PWSs should also report the sample volume spiked and estimated number of oocysts spiked. These data are not applicable to monitoring samples.

EPA recommends that these data elements be reported by submitting a completed sample collection form, laboratory bench sheet, and *Cryptosporidium* report form for each sample. Example bench sheets and report forms are provided as attachments in the *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*, available for download from http://www.epa.gov/safewater/lt2/index.html. Sample documentation forms that are different from these examples, but that contain the minimum required data elements listed above, may be acceptable.

2.2.1.3 Supporting Quality Control Information

The data package should include a signed letter from the laboratory certifying that all method-required quality control elements (including sample temperature upon receipt, ongoing precision and recovery and method blank results, holding times, and positive and negative staining controls) were performed at the required frequency, and were acceptable for every monitoring and MS sample submitted with the package (however, the actual MS sample results are not required to meet the methods' MS QC acceptance criteria). The letter should include a list of the applicable monitoring and MS samples, and the corresponding OPR and method blank sample ID for each.

Alternately, the PWS may include the bench sheet and *Cryptosporidium* report form (or comparable detailed data reporting forms) for each OPR and method blank sample associated with the field and MS samples in the grandfathered data package. If this option is selected, the letter from the laboratory still should certify that sample temperature upon receipt, holding times, and positive and negative staining controls were acceptable for all samples. (The letter is not necessary if detailed data reporting forms containing this information are submitted for the field and MS sample results.)

2.2.2 Schedule for Submission of Grandfathered Data

EPA's current intent is that PWSs with at least 2 years of grandfathered data at the time of LT2 rule promulgation and that intend to use these data in lieu of monitoring under the LT2 rule (*i.e.*, do NOT intend to conduct additional monitoring) should submit these data to EPA within 2 months following LT2 rule promulgation (currently planned for mid- or late 2004). EPA plans to notify these PWSs within 4 months following LT2 rule promulgation as to whether their data are sufficient for bin classification [40 CFR part 141.708 (f)].

PWSs with fewer than 2 years of grandfathered data at the time of LT2 rule promulgation, or that have at least 2 years of grandfathered data but intend to conduct monitoring under the LT2 rule, should submit these data to EPA within 8 months of LT2 rule promulgation (which provides the systems with 2 months to review data from the last potential historical sampling event). Data collected when LT2 rule monitoring begins (6 months after promulgation) will be submitted through the LT2 Data Collection System [40 CFR part 141.708 (g)].

Under the Agreement, PWSs should conduct monitoring under the LT2 rule unless notified in writing by EPA that they have 2 years of acceptable data.

2.2.3 Procedures for Submission of Grandfathered Data

EPA does not intend to formally accept grandfathered *Cryptosporidium* data until the LT2 rule is finalized. The final rule will include procedures for submission of grandfathered data.

2.3 Checklists for Grandfathering *Cryptosporidium* Data

To help PWSs interested in monitoring for *Cryptosporidium* before LT2ESWTR apply the information provided in this guidance, two checklists have been developed. The "Checklist for Beginning Grandfathered *Cryptosporidium* Monitoring"(**Appendix A**) is designed to be used by PWSs to check their monitoring plans against this guidance document before proceeding with monitoring. The "Checklist for Submitting Grandfathered *Cryptosporidium* Data" (**Appendix B**) is designed to be used by PWSs to check their data package against the information in this guidance document before submitting the data package to EPA for review.

SECTION 3: UNDERSTANDING *CRYPTOSPORIDIUM* ANALYSES

The LT2 rule requires the use of EPA Method 1622 or EPA Method 1623 for *Cryptosporidium* monitoring [40 CFR part 141.705 (a)]. This section provides utility personnel unfamiliar with *Cryptosporidium* sample analyses with information on how the analyses are performed and on the quality control (QC) measures the laboratory uses to verify data quality.

3.1 Summary of EPA Methods 1622 and 1623

EPA Methods 1622 and 1623 resulted from an EPA effort initiated in 1996 to identify new and innovative technologies for analysis of source water samples for *Cryptosporidium* and *Giardia*. The methods are identical in most respects, generally differing only in the addition of *Giardia* antibodies in EPA Method 1623's purification and staining procedures. Both EPA Methods 1622 and 1623 were subjected to interlaboratory validation studies using various source waters, and used in a national survey of 87 surface water plants (the Information Collection Rule Supplemental Surveys) to provide EPA with a realistic indication of how the methods would perform when they were used in the monitoring study (Reference 9.1).

Both EPA Methods 1622 and 1623 also were developed as "performance-based" methods. The methods include quantitative criteria requirements (minimum recovery and maximum variability) for initial and ongoing QC samples. These criteria are used to verify acceptable laboratory performance using the version of the method originally validated or to determine whether a modified version of the method performs acceptably.

In EPA Methods 1622 and 1623, the following steps are performed:

- **Filtration.** The sample is filtered in the field or in the laboratory using one of the filters approved for use with EPA Methods 1622 and 1623:
  - Pall Gelman Envirochek™ capsule filter
  - Pall Gelman Envirochek™ HV capsule filter
  - IDEXX Filta-Max™ foam filter

  The oocysts, cysts, and extraneous materials are retained on the filter.

- **Elution.** Materials on the filter are removed by elution with an aqueous buffered salt detergent solution. This elution process is performed differently for each filter:
  - For the Pall Gelman Envirochek™ and Envirochek™ HV filters, elution is performed by filling the capsule with elution buffer, attaching the filter to a "wrist shaker" type lab shaker, and allowing the filter to shake for 5 minutes at a time in three different orientations.
  - For the IDEXX Filta-Max™ filter, the elution technique differs by laboratory. Some laboratories may add the foam filter and elution buffer to a manual plunger chamber to expand the foam filter and flush any oocysts out of the pores in the foam. Other laboratories may add the foam filter rings and elution buffer to a stomacher bag and use a stomacher to elute the filter.

  - For the Whatman CrypTest® filter, elution is performed by adding elution buffer to the filter housing and using sonication and pressurized backwashing to separate oocysts from the filter fabric.

- **Concentration.** After the filter is eluted, the eluate is centrifuged to concentrate the eluted particles into a "packed pellet" at the bottom of the centrifuge tube. This packed pellet is measured by the laboratory analyst. If the pellet volume is ≤ 2 mL (and 10 L was filtered) the entire sample must be analyzed. If the pellet volume is > 2 mL, only 2 mL is required to be analyzed under the LT2 rule (although the utility may request that more be analyzed).

- **Aspiration and resuspension.** The analyst aspirates the supernatant from the top of the packed pellet to minimize the total sample volume, and resuspends the pellet material by vortexing the sample. The analyst measures the total resuspended concentrate volume. If the packed pellet volume was > 2 mL, and the entire sample volume will not be analyzed, only a portion of the concentrate volume will be processed through the remainder of the method. By dividing the concentrate volume processed through the remainder of the method by the total concentrate volume, the laboratory can determine what percent of the sample volume filtered was actually analyzed. By multiplying this percentage by the sample volume filtered, the laboratory can determine the volume analyzed.

- **Purification.** Magnetic beads conjugated to anti-*Cryptosporidium* antibodies are added to the sample concentrate and allowed to mix with the sample, where they attach themselves to any oocysts present. The magnetized oocysts are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. The magnetic bead complex is then detached from the oocysts.

- **Application of the purified sample to a slide.** After immunomagnetic separation, the purified sample is applied to a microscope slide.

- **Drying the sample.** The sample is dried to the slide for several hours to several days to allow the sample to be stained and rinsed without loss of organisms.

- **Staining the sample.** Two stains are added to the sample before it is examined to help the analyst identify any *Cryptosporidium* that may be present. The oocysts and cysts are stained on the slide with fluorescently labeled monoclonal antibodies and 4',6-diamidino-2-phenylindole (DAPI).

- **Examining the sample.** During microscopic examination of the slide, three evaluation techniques are required by EPA Methods 1622 and 1623 to determine whether an object is a *Cryptosporidium* oocyst. (Guidance on interpreting examination results is provided in Section 7.3.)

3.2 *Cryptosporidium* Laboratory Quality Control

As required by both EPA Method 1622/1623 and the Laboratory QA Program, laboratories approved to perform *Cryptosporidium* analyses for the LT2 rule must perform specific quality control (QC) steps during sample analyses to demonstrate that data are reliable [40 CFR part 141.705 (a)(3)]. These QC steps are described below, in Sections 3.2.1 - 3.2.7.

3.2.1 Initial Precision and Recovery Test

Before performing field sample analyses using EPA Methods 1622 or 1623, the laboratory must demonstrate acceptable performance. This is demonstrated by the initial precision and recovery (IPR) test, which consists of four reagent water samples spiked with 100 to 500 oocysts. The results of the four analyses are used to calculate the average percent recovery and the relative standard deviation (RSD) of

the recoveries for *Cryptosporidium*. For EPA Methods 1622/1623, the mean *Cryptosporidium* recovery must be in the range of 24% to 100% and the RSD of the four recoveries must be less than 55%. If more than one process will be used for filtration and/or separation of samples, a separate set of IPR samples must be analyzed for each process.

3.2.2 Method Blank Test

The method blank test in EPA Method 1622/1623 consists of analysis of an unspiked reagent water sample to demonstrate freedom from contamination. One method blank sample must be analyzed each week or every 20 samples, whichever is more frequent. If more than one process will be used for filtration and/or separation of samples, a separate method blank must be analyzed for each process. If one or more *Cryptosporidium* oocysts are found in a blank, analysis of additional samples is halted until the source of contamination is eliminated and a blank shows no evidence of contamination.

3.2.3 Ongoing Precision and Recovery Test

The ongoing precision and recovery (OPR) in EPA Method 1622/1623 entails analysis of a reagent water sample spiked with 100 to 500 oocysts to demonstrate ongoing acceptable performance. One OPR sample must be analyzed each week or every 20 samples, whichever is more frequent. If more than one process will be used for filtration and/or separation of samples, a separate OPR sample must be analyzed for each process. OPR samples must be analyzed before any monitoring samples are processed for each batch to verify acceptable performance. OPR *Cryptosporidium* recovery must be in the range of 11% to 100% to be considered acceptable.

3.2.4 Holding Time Requirements

During *Cryptosporidium* analyses for the LT2 rule, sample processing should be completed as soon as possible by the laboratory. The laboratory should complete sample filtration, elution, concentration, purification, and staining the day the sample is received wherever possible. However, the laboratory is permitted to split up the sample processing steps if processing a sample completely in one day is not possible. If this is necessary, sample processing can be halted after filtration, application of the purified sample onto the slide, or staining.

The following holding times must be met for samples analyzed by EPA Methods 1622/1623 during the LT2 rule:

- **Sample collection and filtration.** Sample elution must be initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field).
- **Sample elution, concentration, and purification.** The laboratory must complete the elution, concentration, and purification in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying.
- **Staining.** The sample must be stained within 72 hours of application of the purified sample to the slide.
- **Examination.** Although fluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination and confirmation should be performed immediately after staining is complete, laboratories have up to 7 days from completion of sample staining to complete the examination and confirmation of samples. However, if fading/diffusion of fluorescien isothiocyanate (FITC) or DAPI staining is noticed, the laboratory must reduce this holding time. In addition, the laboratory may adjust the concentration of the DAPI staining solution so that fading/diffusion does not occur.

3.2.5 Staining Controls

Positive staining controls entail staining and examination of a slide with positive antigen or 200 to 400 intact oocysts to verify that the stain is fluorescing appropriately. These controls are prepared with each batch of slides that are stained. Negative staining controls entail staining and examining a slide with phosphate buffered saline solution to verify that no oocysts or interfering particulates are present.

3.2.6 Proficiency Testing Samples

As part of the Lab QA Program, laboratories must successfully analyze initial proficiency testing (IPT) samples initially, and an ongoing proficiency testing (OPT) samples three times per year. These samples and the Lab QA Program are discussed in more detail in the *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*.

3.2.7 Matrix Spike Samples

The matrix spike (MS) test in EPA Method 1622/1623 entails analysis of a separate sample aliquot spiked with 100 to 500 oocysts to determine the effect of the matrix on the method's oocyst recovery.

One MS sample must be analyzed for every 20 samples from your PWS. The first MS sample should be collected and analyzed during the first sampling event under the monitoring program and at least 12 months must elapse between the first and last MS sample. You should evaluate the MS recoveries, as well as other attributes of sample processing and examination, and work with the laboratory to determine whether sample filtration and processing procedures are working acceptably, or need to be re-evaluated.

If it is not possible to analyze an MS sample for the first sampling event due to laboratory sample processing burden or other reasons, the first MS sample should be analyzed as soon as possible to identify potential method performance issues with the matrix. The requirement that at least 12 months must elapse between the first and last MS sample still applies. For example, if a PWS that is monitoring monthly for 24 months is unable to process an MS sample until the 8th sampling event, due to laboratory sample processing load, the second MS sample can be processed no earlier than the 20th sampling event.

EPA Method 1622/1623 specifies the following additional requirements for MS sample analyses:

- The MS sample volume analyzed must be within 10% of the volume analyzed for the associated field sample.
- The MS sample must be analyzed in the same QC batch as the field sample, using the same method.
- The MS sample must be collected as a split sample or immediately before or after the associated field sample.

Under the LT2 rule, If the volume of the MS sample is greater than 10 L, the system is permitted to filter all but 10 L of the MS sample in the field, and ship the filtered sample and the remaining 10 L of source water to the laboratory to have the laboratory spike the remaining 10 L of water and filter it through the filter used to collect the balance of the sample in the field [40 CFR part 141.705 (a)(2)(ii)].

3.3 Archiving Examination Results

Although not required, laboratories also can archive slides and/or take photographs of slides to maintain for clients. Slides should be stored in a humid chamber in the dark at 0°C to 10°C. An alternative mounting medium also may be used, which may potentially preserve slides longer. Details are provided in the *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule).*

SECTION 4: UNDERSTANDING *E. COLI* ANALYSES

As noted in Section 1, *E. coli* and turbidity data generated under the LT2 rule are used differently for large systems than small systems. *E. coli* and turbidity are reported with *Cryptosporidium* data by large systems to enable EPA to determine whether an *E. coli* trigger level can be established through the microbial index. If a defensible trigger level can be established between *E. coli* concentrations and *Cryptosporidium* levels, small systems will be able to perform less-expensive *E. coli* analyses initially to determine whether more expensive *Cryptosporidium* monitoring is even necessary.

Although *E. coli* data will not be used to determine whether additional treatment is needed for large systems, as *Cryptosporidium* data will, it is nonetheless critical that the large systems generate reliable *E. coli* data to establish relevant trigger levels for use by the small systems. The *E. coli* data generated by small systems will be used to determine whether *Cryptosporidium* monitoring is required, so it is critical that these data be reliable, as well.

This section provides utility personnel unfamiliar with *E. coli* sample analyses with an overview of the methods used under the LT2 rule and the quality control (QC) measures the laboratory uses to verify data quality.

4.1 Summary of LT2 Rule *E. coli* Methods

E. coli sample analyses performed under the LT2 rule must be quantitative; presence/absence *E. coli* results are unacceptable under LT2. The methods described below are approved for the analysis of *E. coli* samples under the LT2 rule [40 CFR part 141.705 (b)].

4.1.1 Most Probable Number (MPN) Methods

4.1.1.1 Standard Methods 9223B: Colilert® and Colilert-18®

Colilert® and Colilert-18® tests are chromogenic/fluorogenic enzyme substrate tests for the simultaneous determination of total coliforms and *E. coli* in water. These tests use commercially available media containing the chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG), to detect total coliforms and the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), to detect *E. coli*. Media formulations are available in disposable tubes for the multiple-tube procedure or packets for the multiple-well procedure. Appropriate preweighed portions of media for mixing and dispensing into multiple-tubes and wells are also available. The use of commercially prepared media is required for quality assurance and uniformity. All tests must be conducted in a format that provides quantitative results [40 CFR part 141.705 (b)].

- **Multiple-Tube.** For the multiple-tube procedure, a well-mixed sample and/or sample dilution/volume is added to tubes containing predispensed media. Tubes are then capped and mixed vigorously to dissolve the media. Alternatively, this procedure can be performed by adding appropriate amounts of substrate media to a bulk diluted sample (with appropriate dilutions for enumeration), then mixing and dispensing into multiple-tubes. A 15-tube MPN should be used to obtain quantitative results. The

number of dilutions/volumes are determined based on the type, quality, and character of the water sample.

- **Multiple-Well.** A multiple-well procedure may be performed with sterilized disposable packets. The commercially available Quanti-Tray® or Quanti-Tray®/2000 multiple-well tests use Colilert® or Colilert-18® media to determine *E. coli* (IDEXX, 1999b,c). In these tests, the packet containing media is added to a 100-mL sample (or appropriate dilutions for enumeration). The sample is then mixed and poured into the tray. A tray sealer separates the sample into 51 wells (Quanti-Tray) or 97 wells (Quanti-Tray/2000) and seals the package.

  After the appropriate sample dilutions/volumes are added, the tubes or trays are incubated at 35°C ± 0.5°C for 18 h when using Colilert-18® or 24 h when using Colilert®. If the response is questionable after the specified incubation period, the sample is incubated for up to an additional 4 h at 35°C ± 0.5°C for both Colilert® tests. Each tube or well is then compared to the reference color "comparator" provided with the media. A yellow color greater or equal to the comparator indicates the presence of total coliforms in the sample, and the tube or well is then checked for fluorescence under long-wavelength UV light (365-nm). The presence of fluorescence greater than or equal to the comparator is a positive test for *E. coli*. If water samples contain humic acid or colored substances, inoculated tubes or wells should also be compared to a sample water blank without Colilert® reagent added. The concentration in MPN/100 mL is then calculated from the number of positive tubes or wells using MPN tables provided by the manufacturer.

4.1.1.2 *Standard Methods* 9221B/9221F: LTB →EC-MUG

The multiple-tube fermentation method for enumerating *E. coli* in water uses multiple-tubes and dilutions/volumes in a two-step procedure to determine *E. coli* concentrations. In the first step, or "presumptive phase," a series of tubes containing lauryl tryptose broth (LTB) are inoculated with undiluted samples and/or dilutions/volumes of the samples and mixed. Inoculated tubes are incubated for 24 ± 2 h at 35°C ± 0.5°C. Each tube then is swirled gently and examined for growth (i.e., turbidity) and production of gas in the inner Durham tube. If there is no growth, acid, or gas, tubes are re-incubated for 24 ± 2 h at 35°C ± 0.5°C and re-examined. Production of growth and gas within 48 ± 3 h constitutes a positive presumptive test for coliforms, which include *E. coli*.

After enrichment in the presumptive medium, positive tubes are subjected to a second step for enumeration of *E. coli*. Presumptive tubes are agitated, and growth is transferred using a sterile loop or applicator stick to tubes containing EC broth supplemented with 4-methylumbelliferyl-β-D-glucuronide (MUG). Inoculated tubes are incubated at 44.5°C ± 0.2°C for 24 ± 2 h in a water bath. All tubes exhibiting growth and gas production are examined for bright blue fluorescence under long-wavelength UV light (366-nm) indicating a positive test for *E. coli*. The density of *E. coli* in MPN/100 mL is then calculated from the number of positive EC-MUG tubes, using MPN tables or formulas. A 15-tube MPN is required under the LT2 Rule.

4.1.2 Membrane Filtration (MF) Methods

4.1.2.1 *Standard Methods* 9222B/9222G: mEndo/LES-Endo→NA-MUG and *Standard Methods* 9222D/9222G: mFC→NA-MUG

These membrane filter methods for enumerating *E. coli* are two-step incubation procedures. First, a sample is filtered through a 0.45 µm filter, then the filter is placed on a pad saturated with mEndo broth or a plate containing mEndo or LES-Endo agar and incubated for 24 ± 2 h at 35°C ± 0.5°C. Pink to red colonies with a metallic (golden-green) sheen on the filter are considered to be total coliforms. If initial determination of fecal coliforms is desired, mFC media can be substituted for mEndo/LES-Endo. Following initial isolation of total coliforms (or fecal coliforms), the filter is transferred to nutrient agar

containing 4-methylumbelliferyl-β-D-glucuronide (NA-MUG) and incubated for 4 h at 35°C ± 0.5°C. Sheen colonies on mEndo or blue colonies on mFC that fluoresce under a long-wavelength UV light (366-nm) are positive for *E. coli*. If high levels of non-*E. coli* total coliforms interfere with the ability to accurately enumerate *E. coli* despite additional dilutions, transfer from mFC or an alternate method (e.g., SM 9213D, EPA Method 1603) should be used.

4.1.2.2 *Standard Methods* 9213D: mTEC

The mTEC agar method is a two-step procedure that provides a direct count of *E. coli* in water, based on the development of colonies on the surface of a membrane filter when placed on a selective nutrient and substrate media. This method originally was developed by EPA to monitor the quality of recreational water. This method was also used in health studies to develop the bacteriological ambient water quality criteria for *E. coli*. In this method, a water sample is filtered through a 0.45μm membrane filter, the filter is placed on mTEC agar (a selective primary isolation medium), and the plate is incubated first at 35°C ± 0.5°C for 2 h to resuscitate injured or stressed bacteria and then at 44.5°C ± 0.2°C for 22-24 h in a water bath. Following incubation, the filter is transferred to a filter pad saturated with urea substrate medium. After 15 minutes, all yellow or yellow-brown colonies (occasionally yellow-green) are counted as positive for *E. coli* using a fluorescent lamp and either a magnifying lens or a stereoscopic microscope.

4.1.2.3 EPA Method 1603: Modified mTEC

The modified mTEC agar method is a single-step MF procedure that provides a direct count of *E. coli* in water based on the development of colonies on the surface of a filter when placed on selective modified mTEC media. This is a modification of the standard mTEC media that eliminates bromcresol purple and bromphenol red from the medium, adds the chromogen 5-bromo-6-chloro-3-indolyl-β-D-glucuronide (Magenta Gluc), and eliminates the transfer of the filter to a second substrate medium. In this method, a water sample is filtered through a 0.45μm membrane filter, the filter is placed on modified mTEC agar, incubated at 35°C ± 0.5°C for 2 h to resuscitate injured or stressed bacteria, and then incubated for 22-24 h in a 44.5°C ± 0.2°C water bath. Following incubation, all red or magenta colonies are counted as *E. coli*.

4.1.2.4 EPA Method 1604: MI Medium

The MI medium method is a single-step membrane filtration procedure used to simultaneously enumerate total coliforms and *E. coli*. In this EPA-developed method, a water sample is filtered through a 0.45-μm membrane filter, the filter is placed on an MI agar or broth plate, and the medium is incubated at 35°C ± 0.5°C for 24 h. If high levels of non-*E. coli* total coliforms interfere with the ability to accurately enumerate *E. coli* despite additional dilutions, an alternate method (e.g., SM 9213D, EPA Method 1603) should be used.

E. coli colonies exhibit a blue color and also may fluoresce under a long-wavelength UV light (366-nm). If desired, the plates can also be observed under long-wavelength UV light (366-nm) for the presence of total coliform species that fluoresce. Because the blue color from the breakdown of indoxyl-β-D-glucuronide (IBDG) can mask fluorescence, non-fluorescent blue colonies are included in the total coliform count. Water samples with high turbidity can clog the membrane filter, interfering with filtration and potentially interfering with the identification of target colonies.

4.1.2.5 m-ColiBlue24® Broth

This broth method is a single-step MF test for enumerating total coliforms and *E. coli*. As with NA-MUG, modified mTEC, and MI media, the selective identification of *E. coli* is based on the detection of the β-glucuronidase enzyme. The test medium includes the chromogen 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (BCIG or X-Gluc). The chromogen BCIG is hydrolyzed by β-glucuronidase, releasing an insoluble indoxyl salt that causes the colonies to exhibit a blue color. M-ColiBlue24® broth is a commercially available format of this method and contains a nutritive lactose-based medium containing inhibitors to eliminate the growth of non-coliforms. With m-ColiBlue24® broth, a water sample is filtered through a 0.45μm membrane filter, and the filter is transferred to a plate containing an absorbent pad saturated with m-ColiBlue24® broth. The filter is incubated at 35°C ± 0.5°C for 24 h and examined for colony growth. The presence of *E. coli* is indicated by blue colonies. The presence of total coliforms (non-*E. coli*) is indicated by red colonies. If enumeration of total coliforms is desired, blue and red colonies should be included in the total coliform count. If high levels of non-*E. coli* total coliforms interfere with the ability to accurately enumerate *E. coli* despite additional dilutions, an alternate method (e.g., SM 9213D, EPA Method 1603) should be used.

4.2 *E. coli* Laboratory Quality Control

E. coli sample results reported under the LT2 rule should meet the quality control (QC) specifications set forth in the approved versions of the methods described above. Sections 4.2.1 - 4.2.7 describe quality control specifications for *E. coli* analyses performed under the LT2 rule. This guidance is provided to help summarize the QC specifications in the methods and does not substitute for or alter the method specifications. Sample results that do not meet these specifications are not considered valid, and cannot be reported under the LT2 rule. Additional information on the QC specifications is available in Section 4.2 of the *Microbial Laboratory Guidance Manual for the Long-Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*.

4.2.1 Dilution/Rinse Water Sterility Check

Each batch (or lot, if commercially prepared) of dilution/rinse water should be checked for sterility by adding 50 mL of water to 50 mL of a double-strength non-selective broth (e.g., tryptic soy, trypticase soy, or tryptose broth). Incubate at 35°C ± 0.5°C, check for growth after 24 hours and 48 hours (or for the longest incubation time specified in the method), and record results. The dilution/rinse water batch should be discarded if growth is detected.

4.2.2 Media Sterility Check

To test sterility of newly prepared media prior to the analysis of field samples, incubate one plate per each media batch at the appropriate temperature for 24 and 48 hours (or for the longest incubation time specified in the method) and observe for growth. If any contamination is observed, determine the cause, correct, and reject any data from samples tested with the media.

4.2.3 Positive/Negative Controls

For each new lot or batch of medium, check the analytical procedures and integrity of the medium before use by testing with known positive and negative control cultures. Laboratories using commercially-prepared media with manufacturer shelf-lives of greater than 90 days should run positive and negative controls each quarter in addition to running the batch/lot-specific controls and sterility checks. Laboratories are encouraged to perform positive and negative control tests each day that field samples are analyzed. Positive and negative controls should be chosen based on the method-specific requirements. For example if a 44.5°C water bath is not required by the method, it is not necessary to include *Enterobacter aerogenes* as a negative control.

4.2.4 Media Storage

The following media storage specifications should be met for *E. coli* analyses:

- Agar plates may be held for up to 2 weeks at 1°C to 5°C in plastic bags or containers. Protect media containing dyes from exposure to light.
- Broth in loose fitting caps (e.g., snap caps) should be stored at 1°C to <30°C for no more than 2 weeks
- Broth in tight fitting caps (e.g., screw caps) should be stored at 1°C to <30°C for no longer than 3 months
- All media should be at room temperature prior to use
- Media exhibiting growth or gas should be discarded

4.2.5 Filtration Unit Sterilization

Membrane filter equipment should be autoclaved before the beginning of a filtration series. A filtration series ends when 30 minutes or longer elapses after a sample is filtered. Ultraviolet (UV) light (254 nm) may be used to sanitize equipment (after initial autoclaving for sterilization), if all supplies are pre-sterilized. UV light can also be used to reduce bacterial carry-over between samples during a filtration series. The UV lamp should be tested quarterly with a UV light meter or an agar plate. Appropriate corrective actions should be taken, if necessary.

4.2.6 Preparation Blanks

Preparation blanks should be analyzed to detect potential contamination of dilution/rinse water during the course of analyses.

4.2.6.1 Membrane Filter Preparation Blank

If membrane filtration is used, an MF preparation blank is performed at the beginning and the end of each filtration series by filtering 20-30 mL of dilution water through the membrane filter and testing for growth. If the control indicates contamination with the target organism, all data from affected samples should be rejected. A filtration series ends when 30 minutes or more elapse between sample filtrations.

4.2.6.2 Most Probable Number Preparation Blank

EPA recommends that a volume of sterilized, buffered water be analyzed exactly like a field sample each day samples are analyzed. The preparation blank should be incubated with the sample batch and observed for growth of the target organism. If the control indicates contamination with the target organism, all data from affected samples should be rejected.

4.2.7 Verification

Verification specifications are detailed in the Certification Manual (Reference 9.3), Standard Methods (Reference 9.4), and **Appendices J** through **L** of the *Microbial Laboratory Guidance Manual for the Long-Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*.

SECTION 5: CONTRACTING FOR *CRYPTOSPORIDIUM* LABORATORY SERVICES

Although many public water systems (PWSs) have established procedures and policies governing the purchase of services and supplies, these procedures seldom lend themselves to the purchase of analytical services. This section provides a basic framework for defining the technical and contractual requirements associated with purchasing laboratory services for *Cryptosporidium* analyses for the LT2 rule, awarding contracts, and working with a contract laboratory.

Successfully contracting for *Cryptosporidium* laboratory services for LT2 rule monitoring relies on the following steps:

Step 1: Define the scope of your analytical requirements to develop a detailed contract and standardized bid sheet

Step 3: Solicit qualified laboratories

Step 4: Award contracts to a primary laboratory and a backup laboratory

Step 5: Work closely with your laboratory before monitoring begins and maintain communications throughout monitoring

Each of these general steps, and details on the activities associated with each, are discussed in Sections 5.1 through 5.5.

5.1 Defining Your Needs and Developing a Contract

The first step in developing an analytical services contract for *Cryptosporidium* analyses for LT2 rule monitoring is identifying the *"who," "what," "when,"* and "*how"* of the project for your system (the *"why"* is the LT2 rule itself). A well-written contract will address each of these issues, as well as the administrative issues, such as laboratory payments and adjustments.

The best way to ensure that you get the data you need for LT2 rule *Cryptosporidium* monitoring within the required time period is to specify your requirements *in detail* in the contract. A well-written contract can minimize or eliminate many common problems in procuring analytical services, and enable you to collect reliable and timely results.

Recommendations on the factors to consider in defining the scope of the services you need, and the information you should be sure to include in your contract are provided below.

5.1.1 Client Information

"Who" defines your PWS to the laboratories that you would like to submit bids for the project. Will you be contracting for laboratory services for a single plant or will this contract require *Cryptosporidium* analyses to fulfill monitoring requirements for multiple plants in a system?

> ☞ *Clearly identify in your contract the name and identification number of your PWS, as well as the name(s) and identification number of the facility(ies) for which samples need to be analyzed. This information ultimately will be used to identify your samples in the LT2 Data Collection System, and the laboratory you use for Cryptosporidium sample analyses will need to know this information. (Alternately, you can provide this information after award to the awarded laboratory only.)*

5.1.2 Sample Information

"What" describes the samples to be analyzed. As noted in Sections 5.1.2.1 through 5.1.2.5, this encompasses a variety of factors, each of which needs to be evaluated and defined before you develop your contract.

5.1.2.1 Number of Samples

What is the total number of samples the laboratory will need to analyze? This total includes not only routine monitoring samples (field samples), but also the matrix spike (MS) samples (Section 3.2.7) that are required at a frequency of 1 per 20 field samples. Field samples and MS samples are considered "billable" samples (sample analyses for which the laboratory will be paid their per-sample cost). Internal laboratory quality control (QC) samples, such as method blanks and ongoing precision and recovery (OPR) samples should be considered "unbillable" samples—sample analyses that are required, but apply to multiple PWS clients. Rather than charging clients for these samples directly, laboratories typically will amortize the costs of these samples across billable samples.

If a sample is collected and sent to the laboratory, but cannot be submitted under the LT2 rule because of a problem unrelated to laboratory performance (such as shipping delays that violate the sample holding time), your PWS will be required to collect a "make-up" sample (see Section 6.3 for details). You should add, as an option to be exercised at your direction in such an event, two additional sample analyses to the total.

> ☞ *Clearly indicate in your contract the total number of: (1) field samples and (2) MS samples that the laboratory will be required to analyze. Add two additional, optional, sample analyses to be exercised if "make-up" samples are required due to problems unrelated to laboratory performance.*

5.1.2.2 Type of Samples

Will your PWS collect and ship bulk water samples to the laboratory for filtration and processing or will your PWS filter samples on-site and ship the filter to the laboratory? Shipping and analytical costs are likely to be lower if you filter your samples on-site, but you will need to purchase or rent sample filtration equipment (see Section 6.4 for details) and have staff trained to use the required procedures or pay for the laboratory or another firm to perform these tasks.

☞ *Clearly specify in the SOW whether the laboratory will receive bulk water samples or filtered samples. If filtered samples will be sent, indicate which filter you will use (see Section 5.1.4.2).*

If you will be filtering on-site, and will be using your own equipment to filter the samples, you should consider purchasing filters directly from the vendor, rather than from the laboratory, to reduce costs. (Additional information on filtering samples on-site and purchasing filters is provided in Section 6.4.2).

☞ *If your PWS will be purchasing filters directly, specify this in the contract, so the laboratory knows not to include this in their per-sample price.*

5.1.2.3 Anticipated Sample Volume

The LT2 rule will require that at least 10 L be analyzed for each sample (with some exceptions - see Section 6.1) [40 CFR part 141.705 (a)(1)]. Will your PWS collect 10-L samples or collect higher-volume samples, such as 50-L samples? If your PWS will be shipping bulk water samples to the laboratory, greater sample volumes will result in higher shipping costs and will likely result in higher analytical costs. If your PWS will be filtering samples on-site, and shipping filters to the laboratory, the sample volume should not affect shipping or analytical costs, but the greater sample volumes filtered may result in higher packed pellet volume and multiple subsamples (Section 5.1.2.4).

☞ *Clearly indicate in your contract the volume you anticipate collecting for each sample.*

5.1.2.4 Subsamples and Filter Clogs

As noted in Section 3.1, additional steps are required at the laboratory for samples that generate a larger packed pellet volume than can be processed as one sample through the method's purification step. Specifically, the laboratory will need to process the packed pellet from the sample as two or more "subsamples" through the remainder of the method (purification, staining, and examination) to meet LT2 rule sample volume analysis requirements. If a sample clogs before 10 L have been filtered, at least two filters must be used to meet LT2 rule sample volume analysis requirements [40 CFR part 141.705 (a)(1)].

If the source water(s) to be monitored by your PWS are characterized by high turbidity, some of your samples may need to be processed as multiple subsamples or may require two filters to enable you to meet LT2 rule monitoring requirements. Even if your source water(s) typically is characterized by low turbidity, you should allow for the possibility that some samples may result in larger packed pellet volumes on occasion. By including this in the original contract, you will avoid changes to the contract on short notice if subsamples are required during monitoring.

☞ *Clearly indicate in your contract that different sample prices are needed for: (1) full sample analyses, (2) subsample analyses, and (3) extra filters and the cost of analysis of the extra filters.*

5.1.2.5 Extra Services

Will any additional services be required of the laboratory outside of actual sample analyses? Possible services include:

- Sampling kit rental for on-site filtration
- Sample shipping containers
- Sample archiving (laboratories can archive slides and some can take photographs of slides to maintain for clients)

Some of these services may be included in the sample analysis cost by some laboratories. Defining the specific services your PWS will need, and specifying these services clearly in the contract will enable the laboratories to better assess whether the requested services are included in their routine costs or are extra, and respond accordingly.

☞ *Clearly specify in your contract any services required in addition to routine sample analysis.*

5.1.3 Sampling Schedules

"When" refers to your anticipated schedule for shipping samples to the laboratory. Will your PWS begin monitoring before implementation of the LT2 rule with the intent to grandfather some or all of the data or will your PWS monitor according to the rule schedule?

The minimum monitoring frequency for the LT2 rule is once per month [40 CFR part 141.701 (e)]. During LT2 monitoring, will your PWS collect and ship samples once per month, or will you monitor more often?

If at all possible, *do not establish a firm sampling schedule with specific dates at this point*. Most of the laboratories available to perform *Cryptosporidium* analyses have multiple PWS clients and need to evenly distribute their sample load within each week and across weeks in a month to meet holding time requirements. Rather than dictating a sample collection schedule to the laboratory–and potentially discouraging laboratories from bidding on the work or risk violating holding times during monitoring—work with the awarded laboratory to establish a schedule that is will comply with LT2 rule requirements and is mutually acceptable to your PWS and the laboratory.

☞ *Indicate in your contract the month that you plan to begin monitoring and whether you will be monitoring on a monthly or more frequent basis. If possible, do not specify actual sample collection dates and days during the week; work with the awarded laboratory to establish a schedule that meets your needs and does not cause problems for the laboratory.*

5.1.4 Analytical Methodology

"How" describes the analytical method that the laboratory will use. This involves two sets of options: which method to use (EPA Method 1622 or EPA Method 1623) and which filter to use, regardless of method. It also refers to the QC requirements that must be met during sample processing and analysis.

5.1.4.1 EPA Method 1622 Versus EPA Method 1623

Will your PWS monitor for *Cryptosporidium* only or *Cryptosporidium* and *Giardia*? Most laboratories analyze samples for both *Cryptosporidium* and *Giardia* using EPA Method 1623. If EPA Method 1623 is used by the laboratory to analyze your LT2 rule samples, only *Cryptosporidium* data need to be submitted. If *Giardia* data are collected, they do not need to be submitted to EPA.

Your contract should specify that EPA Method 1622 be used only if you are interested in monitoring for *Cryptosporidium* only (this method only detects *Cryptosporidium*). Although reagent costs for this method are slightly less than for EPA Method 1623, actual sample analysis costs may not be lower because laboratories may not be able to allocate the QC sample costs for this method across as many clients.

5.1.4.2 Filter Options

Although EPA validated EPA Method 1622 and EPA Method 1623 using one filter type, modified versions of the methods using alternate filter options have been approved by EPA since validation. The following available filters are considered acceptable by EPA for use with EPA Methods 1622 and 1623:

- Original Pall Gelman Envirochek™ capsule filter
- IDEXX Filta-Max™ foam filter
- Pall Gelman Envirochek™ HV capsule filter

Unless your PWS has experience with *Cryptosporidium* sampling, and a basis for requesting a specific filter type, you should indicate in the contract that all are acceptable.

If your PWS has experience monitoring for *Cryptosporidium* and has a filter preference, you will need to indicate this to the laboratories interested in bidding on the project, as not all laboratories are approved by EPA through the Lab QA Program to perform all versions of the methods.

> ☞ *If your PWS has experience with Cryptosporidium sampling and would like analyses performed using a specific filter, clearly indicate this in the contract. Otherwise, do not specify a filter type.*

5.1.4.3 Quality Control Requirements

Although EPA Methods 1622 and 1623 specify the QC requirements that must be met during performance of the method, your contract should reiterate that the following QC tests must be performed at the required frequency during processing and analysis of your samples:

- Method blank test (Section 3.2.2)
- Ongoing precision and recovery (OPR) test (Section 3.2.3)
- Holding time requirements (Section 3.2.4)
- Staining controls (Section 3.2.5)

None of these QC measures should be billable, however. As noted above, in Section 5.1.2.1, the costs for the method blank, OPR, and staining control tests should be amortized by the laboratory across the cost of monitoring samples for all of their clients.

☞ *Reiterate in the contract that method blanks, OPRs, and staining controls must be performed at the frequency required in the method, and that all holding times must be met.*

5.1.5 Data Deliverables and Other Contract Issues

In addition to the *"who," "what," "when,"* and *"how"* questions that need to be addressed by the contract, you also will need to provide details on data delivery, adjustments for lateness, and sample reanalysis cost issues. These issues are discussed in Sections 5.1.5.1 through 5.1.5.5.

5.1.5.1 Data Submission

EPA has developed the web-based LT2 Data Collection System to allow laboratories to report data to PWSs electronically and allow PWSs to verify the data electronically before submitting the monitoring results to EPA. This reporting process is summarized in Section 7.2 for *Cryptosporidium* data, and discussed in detail in the *Users' Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) Data Collection System*. The laboratory, at a minimum, will need to submit the results for each *Cryptosporidium* monitoring sample to you electronically. (Although your PWS also could enter these data, based on hardcopy results from the laboratory, this is strongly discouraged, as the potential for error increases when personnel unfamiliar with the generation of the data for a sample enter these data into the LT2 Data Collection System.)

☞ *Clearly indicate in your contract that the laboratory is required to enter Cryptosporidium monitoring results for your samples into the LT2 Data Collection System.*

5.1.5.2 Hardcopy Data Deliverables

Note: *If you do not intend to review all of the raw data generated by the laboratory, this section is not relevant, and can be ignored.* If your PWS does intend to review all of the raw data associated with your LT2 samples (discussed in Section 7), you should request copies of the forms used by the laboratory to record sample measurements, sample processing times, and sample examination results, as well as information on the QC samples associated with your monitoring sample. (If your PWS will store and maintain all sample results, rather than the laboratory, then the original forms should be requested.)

Suggestions for the materials that should be requested include the following:

- **Sample result summary sheet**, which should include the following:
  - Monitoring sample identification information
  - Monitoring sample result, in oocysts/L
  - Laboratory quality control batch associated with the sample
  - ID number and result for the ongoing precision and recovery (OPR) sample analyzed for this QC batch
  - ID number and result for the method blank sample analyzed for this QC batch
- **LT2 sample collection form** initiated by your utility and completed with sample receipt information by the laboratory

- **Method 1622/1623 Bench Sheet** with raw data associated with the monitoring sample (and MS sample, if applicable)

- **Method 1622/1623 *Cryptosporidium* Slide Examination Form** with raw data for the monitoring sample (and MS sample, if applicable)

- **Laboratory comments.** If the laboratory provided comments on the sample analyses or results that require follow-up, contact the laboratory to discuss, if necessary. Comments may include any applicable data qualifiers. The following is a list of potential data qualifiers:
  - The recovery for the associated ongoing precision and recovery (OPR) sample did not meet method requirements
  - Oocysts were detected in the method blank
  - Positive and negative staining controls were not acceptable or not examined
  - Method holding times were not met
  - Sample arrived at the laboratory in unacceptable condition

☞ *If you need the laboratory to submit hardcopy results (this is not necessary, unless you intend to review all of the raw data), clearly indicate in your contract the materials that are required.*

5.1.5.3 Data Turnaround Requirements

Under the LT2 rule, PWSs are required to submit data no later than 10 days after the end of the first month following the month when the sample is collected (approximately 40 to 70 days after sample collection, depending on when during the month the sample is collected) [40 CFR part 141.707 (d)]. For example, if a sample is collected on March 17, data must be submitted by May 10.

The required data turnaround must be stated clearly in the contract. This turnaround time should be expressed in calendar days (not working days), and should start from the sample collection date. The data turnaround time calculations should consider the day that the sample is collected “day zero," and the following day as "day one." (Data turnaround times in analytical contracts typically start from the receipt of the sample at the laboratory, but calculating it from the sample collection date is more logical in this case because the LT2 rule’s data submission requirements are based on sample collection date.)

If the data turnaround time starts from sample collection, rather than sample receipt by the laboratory, this turnaround should accommodate the potential for shipping delays that will be outside of the laboratory’s control. As a general rule, the data turnaround time should not be shorter than the sum of the maximum holding times in the method—15 days. This includes up to 4 days between sample collection and initiation of the elution step, which effectively is the maximum time for any shipping delay, as samples received more than 4 days after collection will not be valid, and cannot be submitted through the LT2 Data Collection System.

Using the 15 days allowed for sample analysis by the methods (plus additional time to compile the data package and mail the results) as the shortest realistic turnaround time, determine when you will actually need the results. The same turnaround time can be specified for both submission of electronic data and receipt of hardcopy materials.

Do not specify a data turnaround time shorter than you really need, as it may increase the per-sample price quoted by the laboratories. This turnaround time should be short enough to provide time to carefully evaluate the results before they must be submitted to EPA, but long enough that it does not unreasonably burden the laboratory and potentially increase the per-sample quotes you receive when you solicit the project.

> ☞ *Specify in the contract the data turnaround requirement for electronic and hardcopy submission of data. This turnaround time should be calculated as the time between sample collection and receipt of the hardcopy data by your PWS.*

5.1.5.4 Liquidated Damages and Penalties

You should consider including penalty or damage clauses in your contracts as incentives to preclude laboratories from submitting data late or performing analyses improperly. Due to the nature of the services provided, it is often difficult to assess actual damages caused by improperly performed analyses. Liquidated damages often are used in analytical services contracts in lieu of actual damages. Liquidated damages typically specify that, if the laboratory fails to deliver the data specified in the deliverables section of the contract, or fails to perform the services within the specified data turnaround time, the laboratory will pay a fixed, agreed, price to compensate the organization to whom the services should have been delivered. For example, some EPA contracts for analytical services specify that the laboratory will pay, as fixed, agreed, and liquidated damages, 2% of the analysis price per calendar day of delay, to a maximum reduction of 50% of the analysis price.

If liquidated damages or penalties are involved, they should (1) be based on actual damage caused (in terms of cost) by each day of lateness, (2) be strong enough to discourage late delivery, and (3) be reasonable enough that they will not discourage laboratories from bidding. If liquidated damages or penalties will be applied to meet the required data turnaround time, this information should be included. The contract should specify that the laboratory will not be charged with liquidated damages when the delay in delivery or performance arises out of causes beyond the control and without the fault or negligence of the laboratory. It also may be necessary to limit damages to a certain dollar value or scope.

Other types of damages that should be considered, and may be included in the contract, include costs for resampling and administrative costs associated with the evaluation and processing of unacceptable data (data that do not meet the requirements specified in the contract or the QC requirements specified in the analytical method).

> ☞ *Clearly indicate in your contract whether liquidated damages will be applied to late data or other problems, how these liquidated damages are calculated, and the limits and conditions associated with the damages.*

5.1.5.5 Re-Analysis Costs

Every laboratory periodically produces data that are associated with unacceptable QC data or are invalid for other reasons. The contract should stipulate that the laboratory will reanalyze samples at no cost to your PWS if the problems are due to laboratory error. If the problems are due to an error outside of the laboratory's control (such as the laboratory's rejection of a sample received at > 10°C that results in resampling by your PWS), the laboratory should not be responsible for the additional costs that may result.

☞ *Clearly indicate in your contract when the laboratory would be required to bear the costs of sample re-analysis costs and when these costs will be borne by your PWS.*

The contract also should state that you have the right to inspect the results, and if they do not meet the requirements in the contract, you have the right to reject the data, returning them to the laboratory without payment. Rejection of data should be based on sound technical review of the results. It also obligates you to make no use of those results without making some payment to the laboratory.

☞ *Clearly indicate in your contract that your PWS has the right to inspect results and reject the results if they do not meet contract requirements.*

5.2 Developing a Bid Sheet

After all project requirements have been established, you should develop a bid sheet to accompany the analytical requirements summary during the solicitation. The bid sheet allows laboratories to submit bids in the same format, making bid evaluations easier, and also helps to clarify the project. Development and use of a bid sheet is recommended regardless of whether your PWS solicits the project competitively to multiple laboratories, or is simply requesting a quote from a laboratory you already know you will be using, as it provides a very clear vehicle for submitting and evaluating costs.

Bid sheets for analytical services typically are formatted as a chart, with analytical requirements along one axis and number of samples and prices along the other.

The bid sheet should include the following information:

- Project identifier (e.g. "LT2 *Cryptosporidium* Monitoring Sample Analyses for [PWS name and/or facility name]")
- Space for laboratory identification information
- Day, date, and time (including time zone) of the bid deadline
- Bid submission information (contact and mailing address, fax number, and/or email address)
- Estimated award date
- Laboratory period of performance (period of time during which the laboratory is obliged to resolve issues associated with analysis of the samples—generally 6 months after shipment of last sample)
- Required delivery date (data turnaround time and the basis of its calculation, such as from collection of each sample)
- Bid validity period (period of time during which bid prices are considered valid—generally 45 days after the bid deadline; if the project is awarded after the period you specify, you must contact bidding laboratories to determine whether their bid is still valid, or needs to be revised)

- A summary of the analytical requirements:
  - Method (e.g., *Cryptosporidium* and *Giardia* by EPA Method 1623)
  - Filter preference, if any (this should not be specified, unless your PWS has experience with *Cryptosporidium*, and a basis for requesting the use of a specific filter; if you know that you will be field filtering using a specific filter, and shipping this to the laboratory, it is important that you specify this)
  - Whether samples will be shipped as filtered samples or bulk water samples
  - Sample volume (e.g., 10 L, 50 L)
- Total number of field samples to be analyzed, plus two extra, in case of "make-up" samples
- Total number of MS samples to be analyzed
- Total number of potential subsamples to be analyzed (expressed as "Up to [no.] subsamples")
  - The number generally should not exceed three per sample
  - If you have high-turbidity water, you may need to specify up to three subsamples for all of your field and MS samples
  - If you have a low-turbidity water, you should specify a minimal number, just in case the need arises

  (These costs would not be incurred unless subsamples actually need to be analyzed)
- Total number of potential extra filters (in case one or more samples clog during LT2 rule monitoring:
  - If you will be shipping bulk samples to the laboratory, express this as "Up to [no.] extra filters/elutions"
  - If you will be filtering samples in the field, but receiving filters from the laboratory, express this as "Up to [no.] extra filters"

  (These costs would not be incurred unless more than one filter actually needs to be used)
- Columns for laboratories to enter per-analysis and total costs

5.3 Soliciting the Contract

Procedures for soliciting and awarding contracts to perform analytical services can vary, depending upon the scope of the project and purchasing requirements within the organization that is issuing the contract. At one end of the spectrum are contracts that are awarded after placing a single phone call and obtaining a quote from a single laboratory. The opposite end of the spectrum are contracts awarded after a competitive solicitation and bidding process involving the distribution of a detailed project description and a formal bid sheet via fax or mail.

5.3.1 Approved Laboratories

Regardless of whether you will be soliciting the project to multiple laboratories or working with a single laboratory (although a backup laboratory is strongly recommended—see below), you will need to limit your laboratories to only those approved by EPA through the Laboratory Quality Assurance Evaluation Program for Analysis of *Cryptosporidium* Under the Safe Drinking Water Act (Laboratory QA Program) (67 *FR* 9731, March 4, 2002). Information on the Laboratory QA Program is posted on

http://www.epa.gov/safewater/lt2/index.html and this program is described in detail in the *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*.

Briefly, the objectives of the program are to evaluate laboratories' capacity and competency to reliably measure for the occurrence of *Cryptosporidium* in surface water using EPA Method 1622/1623. Each laboratory participating in the program is required to complete the following steps to be qualified through this program:

- Acceptably perform initial proficiency testing (IPT) on blind samples
- Participate in an on-site evaluation of their technical, data management, and quality assurance procedures
- Acceptably perform ongoing proficiency testing (OPT) on blind samples every four months

To improve *Cryptosporidium* data quality and consistency during LT2 rule monitoring, EPA requires that only those laboratories approved for *Cryptosporidium* analysis under the Lab QA Program be used for LT2 rule monitoring analyses [40 CFR part 141.706 (a)]. A list of laboratories approved through the Lab QA Program is available from http://www.epa.gov/safewater/lt2/index.html.

5.3.2 Primary and Backup Laboratory Contracts

Because a laboratory's approval status may change during the LT2 rule monitoring period, you should plan to award a primary contract and a backup contract. If no performance problems or other problems are encountered during the LT2 rule monitoring period by the laboratory awarded the primary contract, then this laboratory would provide uninterrupted sample analysis support for the entire monitoring period. However, if the laboratory encountered performance problems and was disapproved, or was otherwise unable to meet contract requirements, your PWS could switch sample analyses to the backup laboratory under the contract you established with this laboratory before monitoring began.

The award of primary and backup contracts should be discussed in the contract solicitation. All other things considered equal, the award for the primary contract could be made to the lowest responsive, responsible bidder and the award for the backup contract could be made to the second lowest responsive, responsible bidder.

5.4 Evaluating Bids

After the laboratories have received the solicitation and submitted their bids, you must evaluate the bids to identify the laboratory that will be awarded the analytical services contract. Specific procedures for evaluating bids may vary, depending upon the requirements of your organization, but the bid evaluation process generally entails evaluation and comparison of each laboratory's proposed cost and capability to meet the analysis requirements.

5.4.1 Identifying Responsive Bidders

You should consult your legal department or purchasing department to identify any applicable requirements for evaluating competitive bids within their organization. At a minimum, however, you should review all bids and recalculate subtotals and totals to ensure that the bidding laboratories did not make any mathematical errors. In addition, you should verify that there are no unacceptable contingencies associated with any of the bids, such as the use of a filter other than the filter that was specified in the contract solicitation. Either eliminate from consideration bids from laboratories that bid with contingencies or contact the laboratory(ies) to discuss the bid and verify that the laboratory cannot perform the specified services.

Of the remaining (responsive) bids, identify the lowest bidder to award the primary contract and the second lowest bidder to award the backup contract. If additional assessments of a laboratory's performance or responsibility are needed, you may want to contact references.

5.4.2 References

If you have not worked with a particular laboratory before and would like to verify that the laboratory will meet your needs throughout the monitoring period, you can ask the laboratory to provide contacts and phone numbers of utility or government clients for which the laboratory has performed *Cryptosporidium* sample analyses or other comparable services.

Questions to ask the references include:

- Did the laboratory provide data by the required due date?
- Were the data provided by the laboratory of acceptable quality and compliant with contract requirements?
- Were laboratory personnel easy to work with when problems arose during all phases of the project, including sample scheduling, sample analysis, and data review? If problems were noted during data review, was the laboratory prompt and responsive in addressing your concerns?
- Do you have any reservations in recommending this laboratory?

5.5 Communicating with the Laboratory

After the analytical services contract is awarded, you should request from the laboratory contact information for the following roles, and provide the laboratory with PWS contacts for the same roles:

- A technical contact for analytical questions or problems
- A sample control contact for shipping delays on the PWS end and sample receipt problems on the laboratory end
- An administrative contact for invoicing and payment

Maintaining communications with the laboratory is critical to identifying and resolving problems quickly and minimizing the need for resampling and reshipments. At a minimum, you should always notify the laboratory of sample shipments and confirm that the laboratory received the sample on time and in acceptable condition.

Although most communications are typically conducted over the phone, these communications also can be conducted via email, which has the added benefit of providing your PWS and the laboratory with a written record of sample receipt confirmations, problem notifications, and problem resolutions.

SECTION 6: COLLECTING AND SHIPPING SOURCE WATER SAMPLES

Large systems (PWSs serving a population of at least 10,000 people) monitoring under the LT2 rule are required to collect and analyze source water samples for *Cryptosporidium*, *E. coli,* and turbidity for a minimum of 2 years. Small systems (PWSs that serve fewer than 10,000 people) are required to monitor their source water for *E. coli* for a minimum of 1 year. A subset of small systems would then be required to conduct *Cryptosporidium* analyses over a 1-year period if they exceed *E. coli* trigger levels (40 CFR part 141.701).

Monitoring requirements for each system size and the schedule for each stage of monitoring is described in **Table 6-1**.

Table 6-1. Summary of LT2 Rule Monitoring Requirements

Public water system size	Monitoring begins	Monitoring duration	Monitoring parameters and sample frequency requirements	
			Cryptosporidium	***E. coli***
Large systems (serving 10,000 or more people)	6 months after promulgation of LT2 rule	2 years[a]	minimum 1 sample/month[c]	minimum 1 sample/month[d]
Small systems (serving fewer than 10,000 people)	30 months (2 ½ years) after promulgation of LT2 rule	1 year[a,b]	see below [§]	1 every 2 weeks
[§] Possible additional monitoring requirement for *Cryptosporidium* *If* small systems exceed *E. coli* trigger levels, *then*. . .				
Small systems (serving fewer than 10,000 people)	48 months (4 years) after promulgation of LT2 rule	1 year	2 sample/month	N/A

[a] PWSs may be eligible to use historical (grandfathered) data in lieu of these requirements if certain quality assurance and quality control criteria are met (see Section 2)

[b] Small systems may be required to monitor for *Cryptosporidium* for 1 year, beginning 6 months after completion of *E. coli* monitoring; *Cryptosporidium* monitoring would be required if the *E. coli* annual mean concentrations exceed 10 *E. coli*/100 mL for systems using lakes/reservoirs or exceed 50 *E. coli*/100 mL for systems using flowing streams

[c] PWSs monitoring for *Cryptosporidium* may collect more than 1 sample per month if sampling is evenly spaced over the monitoring period

[d] Large unfiltered systems are required to conduct source water monitoring that includes *Cryptosporidium* sampling only

N/A = Not applicable. No monitoring required.

6.1 Sample Volumes

Sample volume guidance is provided in Section 6.1.1 for *Cryptosporidium* samples and Section 6.1.2 for *E. coli* samples.

6.1.1 *Cryptosporidium* Samples

Under LT2 rule *Cryptosporidium* sample volume requirements [40 CFR part 141.705 (a) (1)], PWSs are required to analyze, at a minimum, either:

- 10 L of sample, *or*
- 2 mL of packed pellet volume, *or*
- As much volume as two filters can accommodate before clogging (this condition applies only to filters that have been approved by EPA for nationwide use with EPA Method 1622/1623—the Pall Gelman Envirochek™ and Envirochek™ HV filters, or the IDEXX FiltaMax™ foam filter).

The LT2 rule sample volume analysis requirement of 10 L (rather than 10.0 or 10.00 L) accommodates the potential for imprecisely filled sample containers or filters. Sample volumes ≥ ##.5 L would be rounded up and sample volumes ≤ ##.4 L would be rounded down. For example, 9.8 L would be rounded to 10 L, and would meet rule requirements.

Systems may analyze larger volumes than 10 L, and larger volumes analyzed should increase analytical sensitivity (detection limit), provided method performance is acceptable. EPA encourages systems to analyze similar sample volumes throughout the monitoring period. However, data sets including different samples volumes will be accepted, provided the system analyzes the minimum sample volume requirements noted above.

PWSs with highly turbid water may be able to collect the required minimum packed pellet volume by avoiding filtration altogether, and shipping a bulk water sample to the laboratory for centrifugation. The laboratory can mix the sample thoroughly and centrifuge 250-mL or greater aliquot volumes sequentially according to Section 13.2 of Method 1622/1623, until 2 mL of packed pellet volume is generated.

If the PWS encounters variable water quality that clogs the filter unpredictably, the PWSs should routinely bring two filters plus a cubitainer to the sampling point for each sampling event:

- If the water quality allows a full 10 L to be filtered without clogging, the PWS can simply ship the filter to the laboratory and save the remaining materials for subsequent events.
- If the first filter clogs after 5 L or more have been filtered, and the volume is not anticipated to yield 2 mL of packed pellet volume, the PWS should be able to filter the remaining volume through the second filter and ship both filters to the laboratory for processing.

6.1.2 *E. coli* Samples

PWSs should analyze up to 100-mL of sample for LT2 monitoring. EPA recommends that the PWS collect and ship more than 100-mL of sample to ensure sufficient volume for sample analysis is available in the event of spillage at the laboratory. If spillage or leakage occurs during shipment, there is an opportunity for sample contamination to occur and the sample should not be analyzed (see Section 8.3.1). Additional details on sample collection procedures are provided in Section 6.4.3. The capacity of sample containers should be 120-mL (6 oz.) or 250-mL (8 oz.) to allow for sufficient sample volume and at least a 1-inch head space to facilitate mixing of the sample by shaking prior to analysis.

6.2 Sample Collection Location

LT2 rule monitoring is intended to assess the mean *Cryptosporidium* level in the influent to drinking water plants that treat surface water or ground water under the direct influence (GWUDI) of surface water. Generally, monitoring is required for each plant that treats a surface water or GWUDI source. However, where multiple plants receive all of their water from the same influent (e.g., multiple plants draw water from the same pipe), the same set of monitoring results may be applied to each plant. *E. coli* samples should be collected at the same location as *Cryptosporidium* samples.

PWSs are required to collect source water samples for the LT2 rule from the plant intake prior to any treatment [40 CFR part 141.704 (a)]. Guidance on sampling at plants where this may not be feasible, or where other factors, such as the use of multiple sources, need to be addressed, is provided below, in Sections 6.2.1 through 6.2.5.

6.2.1 Plants That Do Not Have a Sampling Tap Located Prior to Any Treatment

Plants in this situation should pursue one of the following options:

- Manually collect source water samples as close to the intake as is feasible, at a similar depth and distance from shore.
- Establish a sampling location prior to treatment
-

6.2.2 Plants That Use Different Water Sources at the Same Time

This includes multiple surface water sources and blended surface water and ground water sources. Plants in this situation should pursue one of the following options:

- If there is a sampling tap where the sources are combined prior to treatment, the sample should be collected from the tap.
- Samples can be manually collected at each source near the intake on the same day and composited into one sample. The volume of sample from each source should be weighted according to the proportion of that source used by the plant. For example, if a plant has two sources and 75% of the drinking water is from Source A and 25% is from Source B, then for a 10-L sample, 7.5 L would be collected from Source A and combined with 2.5 L collected from Source B. Compositing of samples should reflect plant operation at the time the sample is collected and may change during the monitoring period.

- Separate samples can be manually collected at each source near the intake on the same day and analyzed independently. The results would then be used to calculate a weighted average of the analysis results. The weighted average would be calculated by multiplying the analysis result for each source by the fraction of the source contribution to total plant flow at the time the samples were collected, and then summing these values. For example, if a plant has two sources and 75% of the drinking water is from Source A and 25% is from Source B, then one sample would be collected from each source and analyzed independently. If the concentration of oocysts for the sample from Source A was 5 oocysts/L and the concentration of the sample from Source B was 2 oocysts/L, the final result for the plant for this sampling event would be 4 oocysts/L ([5 oocysts/L x 0.75] + [1 oocyst/L x 0.25]).

6.2.3 Plants That Use Presedimentation

For these plants, source water samples must be collected after the presedimentation basin but before any other treatment [40 CFR 141.704 (b)]. Use of presedimentation basins during monitoring should be consistent with routine operational practice and should be documented. For systems taken samples after

presedimentation basin, no "Microbial Toolbox" credits will be allowed for presedimentation, if the plant is classified into a bin that requires additional treatment [40 CFR 141.726 (a)].

6.2.4 Plants That Use Raw Water Off-Stream Storage

For these plants, source water samples must be collected after the off-stream storage reservoir [CFR 141.704 (c)]. Use of off-stream storage during monitoring should be consistent with routine operational practice and should be documented.

6.2.5 Plants That Use Bank Filtration

The correct sampling location for PWSs with plants using bank filtration differs depending on whether the bank filtered water is treated by subsequent filtration for compliance with the Surface Water Treatment Rule (SWTR) [40 CFR 141.704 (c)].

- PWSs using bank filtered water that is treated by subsequent filtration for compliance with the SWTR must collect source water samples from the well (i.e., after bank filtration) but before any other treatment. Use of bank filtration during monitoring should be consistent with routine operational practice and should be documented. Systems collecting samples after a bank filtration process may not receive microbial toolbox credit for the bank filtration [40 CFR 141.726 (c)].

- PWSs using bank filtered water without additional filtration must take source water samples in the surface water source (e.g., the river). Use of bank filtration during monitoring should be consistent with routine operational practice and should be documented.

Before monitoring begins, all plants must establish a source water monitoring schedule, as discussed in Section 6.3.

6.3 Source Water Monitoring Schedule

PWSs are required to collect samples at least monthly and in accordance with a schedule established by the PWS prior to initiation of monitoring. PWSs may collect samples more frequently (e.g., twice-per-month, weekly), provided the same frequency is maintained throughout the monitoring period [40 CFR part 141.701 (e)].

Water treatment plants that use surface water or ground water under the direct influence (GWUDI), but are operated only seasonally (e.g., during times of high-water demand) should monitor at least monthly during the period when the plant is in operation.

Systems regulated under the LT2 rule are required to submit source water monitoring schedule to EPA within 3 months of rule promulgation [40 CFR part 141.703 (a)]. The schedule is entered using the scheduler function within the LT2 Data Collection System. Details on the use of the scheduler are provided in the *Users' Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) Data Collection System*. Systems are required to collect samples within 2 days before or after the dates indicated in their sampling schedules [40 CFR part 141.703 (b)].

The scheduler function will be available for PWSs to establish their LT2 monitoring schedule for a 3-month period, beginning on the date of final rule publication. The use of a predetermined monthly or semimonthly sampling schedule at each PWS during LT2 is designed to capture storm events and other factors that affect water quality on a periodic basis. Because a PWS can potentially bias the results of the monitoring by avoiding sample collection during periods of low water quality, the submission of pre-scheduled sampling dates will be used to assess compliance.

6.4 Sample Scheduling Compliance Issues

Permissible exceptions to the sampling schedule are noted as follows:

- If extreme conditions or situations exist that may pose danger to the sampler, or which are unforeseen or cannot be avoided and which cause the system to be unable to sample in the required time frame, the system should sample as close to the scheduled date as feasible and submit an explanation for the alternative sampling date to EPA concurrent with shipment of the sample to the laboratory.

  EPA will evaluate the explanation and update the schedule in the LT2 Data Collection System, if acceptable, to permit the analytical result to be submitted through the system (results with sample collection dates that do not comply with the schedule entered by the PWS before monitoring began will be rejected from the system).

- Systems that are unable to report a valid *Cryptosporidium* analytical result for a scheduled sampling date due to failure to comply with the analytical method quality control requirements (e.g., sample is lost or contaminated; laboratory exceeds analytical method holding time) must collect a replacement sample within 14 days of being notified by the laboratory that a result cannot be reported for that date. Systems must submit an explanation for the replacement sample with the analytical results. Systems should collect an *E. coli* sample at the same time as the *Cryptosporidium* replacement sample.

Alternative sample collection dates should be timed so as not to coincide with another scheduled *Cryptosporidium* sample collection date. Documentation of alternate sample collection, including the reason, should be provided with the grandfathered data package.

6.4 Sample Collection Guidance

Large plants must begin collecting source water samples 6 months after rule promulgation and small plants must begin 30 months after rule promulgation. Because the LT2 monitoring program is designed to assess source water *Cryptosporidium* and *E. coli* concentrations, not the concentrations of these organisms at points after any treatment, samples must be collected prior to any treatment and where the water is no longer subject to surface runoff during LT2 monitoring (40 CFR part 141.704).

During each of the scheduled sampling events, several actions must be performed in addition to collecting the sample. These actions, and an indication of which plant types each applies to, are summarized in **Table 6-2**.

Table 6-2. Sample Collection Activities Required for Each Plant Type

Action	Large filtered plants	Large unfiltered plants	All small plants	Small plants that exceed the *E. coli* trigger level
Document sample collection information	✓	✓	✓	✓
Collect *Cryptosporidium* sample	✓	✓		✓
Collect *E. coli* sample	✓		✓	
Measure turbidity	✓			
Monitor sample temperature during sample transport	✓[a]	✓[a]	✓[b]	✓

[a] Those utilities with on-site *Cryptosporidium* analytical capabilities will not need to transport samples unless the laboratory is not located in close proximity to the sample collection location

[b] Those small plants with on-site *E. coli* analytical capabilities will not need to transport samples unless the laboratory is not located in close proximity to the sample collection location

Guidance and procedures for each of these sample collection activities is provided in Sections 6.4.1 - 6.4.5, below.

6.4.1 Sample Collection Documentation

The information in **Table 6-3** should be recorded during sample collection to link the monitoring result to the plant, and to provide information required for development of the microbial index.

Table 6-3. Minimum Data Elements to Record During Sample Collection

Sampling Information	Required	Recommended
PWS name		✓
Public Water System Identification (PWSID) number[a]	✓	
Facility name		✓
Facility ID[a]	✓	
Sample collection point name		✓
Sample collection point ID[a]	✓	
Sample collection date[a]	✓	
Source water type[b]	✓	
Requested analysis		✓
Sample collection time (start time for field-filtered samples)		✓
Meter readings *(for field-filtered samples only)*		✓

Sample collection stop time *(for field-filtered samples only)*		✓
Turbidity[b]	✓	

[a] The combination of these elements constitute the unique sample identifier for LT2 monitoring samples
[b] This information should be recorded with the *E. coli* sample collection information, as it will be entered into the LT2 data collection system with the *E. coli* sample results, for use in reassessing the microbial index. It does not need to be reported with the *Cryptosporidium* sample collection information

For samples that are shipped off-site, this information should be documented on an LT2 sample collection form (**Appendix C**), or similar form provided by your contract laboratory. For samples analyzed on-site by your utility's laboratory, this information can be documented in a sampling log book or other standard form used by your utility; the LT2 sample collection form can also be used.

The source water type for the sample will be used to reassess the relationship between *Cryptosporidium* and *E. coli* concentrations (the microbial index discussed in Section 1.5). Sample collection personnel must select from four source water types on the LT2 sample collection form:

- Flowing stream (defined under the LT2 rule as "a course of running water flowing in a definite channel")
- Reservoir/lake (defined under the LT2 rule as "a natural or man made basin or hollow on the Earth's surface in which water collects or is stored that may or may not have a current or single direction of flow")
- Ground water under the direct influence (GWUDI) of flowing stream surface water
- GWUDI of reservoir/lake surface water

The source water type should be selected based on the type of source water that accounts for *the majority of the surface water used as source water at the time of sample collection*. For example, if the plant uses a mix of approximately 55% reservoir/lake water and 45% flowing/stream water, the "reservoir/lake" option should be circled on the LT2 sample collection form.

The majority of source water for plants that use GWUDI is ground water. However, as noted above, the selection of source water type under the LT2 rule is based on the majority of surface water used as source water. As a result, the selection of source water type is based on the type of surface water that accounts for the majority of the influence of the ground water source.

The turbidity of the source water also needs to be measured. *Cryptosporidium* sample collection procedures are discussed in Section 6.4.2; *E. coli* sample collection procedures and turbidity measurement procedures are discussed in Section 6.4.3 and 6.4.4, respectively.

6.4.2 *Cryptosporidium* Sample Collection

Several options are available to the PWS in collecting untreated surface water samples for *Cryptosporidium* analysis, including the following:

- Collection of bulk water samples for shipment to the laboratory for filtration and analysis. A detailed protocol for collecting, packing, and shipping bulk samples is provided as **Appendix D**.
- On-site filtration of water samples using the Pall Gelman Envirochek™ or Envirochek™ HV capsule filter. A detailed protocol for filtering samples on-site from pressurized or unpressurized sources is provided as **Appendix E**.
- On-site filtration of water samples using the IDEXX™ Filta-Max foam filter. A detailed protocol for filtering samples on-site from pressurized or unpressurized sources is provided as **Appendix F**.

Regardless of the procedure used to collect *Cryptosporidium* samples, the sample must be eluted from the filter within 96 hours of sample collection, per EPA Method 1622/1623 (Section 8.2). If this holding time is violated, the laboratory will reject the sample, and your PWS will be required to recollect and reship the sample.

LT2 rule requirement: *Each sample must meet the QC criteria for the methods [40 CFR part 141.705 (a) (3)]. Per EPA Method 1622/1623, samples must be processed or examined within each of the holding times specified by the method (Section 8.2).*

6.4.2.1 Matrix Spike Samples

Method 1622/1623 requires matrix spike (MS) samples to be analyzed at a frequency of 1 MS sample for every 20 monitoring samples from each plant. This frequency translates to the following, for each plant category:

- For large PWSs that perform monthly monitoring for 2 years (resulting in 24 monitoring samples), 2 MS samples must be collected and analyzed
- For large PWSs that perform semi-monthly or more frequent monitoring for 2 years (resulting in 48 or more samples), a minimum of 3 MS samples will be collected and analyzed
- For small PWSs that are triggered into *Cryptosporidium* monitoring and collect semi-monthly samples for 1 year (resulting in 24 samples), 2 MS samples must be collected and analyzed

The MS sample and the associated unspiked sample must be analyzed by the same procedure and the MS sample must be the same volume as the associated monitoring sample. If the volume of the MS sample is greater than 10 L, the system is permitted to filter all but 10 L of the MS sample in the field, and ship the filtered sample and the remaining 10 L of source water to the laboratory to have the laboratory spike the remaining 10 L of water and filter it through the filter used to collect the balance of the sample in the field.

Utilities collecting and shipping bulk water samples for filtration and analysis at the laboratory should split their sample stream and collect the monitoring sample volume and MS sample volume simultaneously.

- The sample stream should be split using flow controllers on both sides of the split to regulate the pressure difference between the side being subjected to filtration (resulting in higher pressure) and the side flowing into a bulk sample container. A mixing chamber (filter housing without filter) can be added immediately upstream from the Y to aid in equalizing the distribution of sample particulates to either side.
- If splitting the sample stream is not practical, the utility should collect the MS sample immediately before or after the monitoring sample.

MS sample results would not be used to adjust *Cryptosporidium* recoveries at any individual source water; but MS results would be used collectively to assess overall recovery and variability for EPA Method 1622/1623 in source water. No resampling would be necessary for MS samples that do not meet Method 1622/1623 recovery guidelines.

LT2 rule requirements: *(1) The MS and field sample must be collected from the same sampling location by splitting the sample stream or collecting the samples sequentially. (2) The volume of the MS sample analyzed must be within 10% of the volume of the field sample analyzed. (3) The MS and field sample must be analyzed by the same procedure [40 CFR part 141.705 (a) (2) (i)].*

6.4.2.2 Purchasing Filters

If one of the field filtration options is used, you may want to consider purchasing filters in bulk from the manufacturer (or the manufacturer's local distributor), as it may be cheaper than purchasing the filters from your *Cryptosporidium* contract laboratory as part of the sampling kit. This approach also provides your PWS with a ready supply of extra filters on-site, if a filter clogs during a sampling event. Plants wishing to explore this option should call one of the contacts in **Table 6-4**.

Table 6-4. Contacts for Filters Approved for Use in EPA Method 1622/1623

Pall Life Sciences (Envirochek™ and Envirochek™ HV capsule filters)	IDEXX (Filta-Max™ foam filters)
www.pall.com/gelman 600 South Wagner Road Ann Arbor, MI 48103 Sales: Phone: (800) 521-1520 ext.2 Fax: (734) 913-6495 Technical Support: Phone: (800) 521-1520 ext.3 Fax: (734) 913-6495	www.idexx.com Sales: Phone: (800) 321-0207 ext.1 Fax: (207) 856-0630 Technical Support: Phone: (800) 321-0207 ext.2 Fax: (207) 856-0630 E-mail: water@idexx.com

The PWS also can purchase and assemble the entire sampling kit and maintain this kit on site, rather than shipping it back and forth between the *Cryptosporidium* laboratory and the plant. If the filters you use have associated shelf lives and storage conditions, ensure that the filters are stored according to the manufacturers' directions and are not used past the specified shelf life.

The components and part numbers for the sampling kit are specified in the individual protocols for each filter. If the sampling kit is maintained on-site by the utility, the utility should use disposable materials wherever possible to mitigate the risk of cross-contamination between samples or sampling events, and must disinfect the non-disposable sampling equipment between uses (if the laboratory provides the sampling kit, this disinfection step is performed at the laboratory.)

Sampling kit cleaning should consist of the following:

- Cleaning equipment by scrubbing with warm detergent solution and exposing to hypochlorite solution (minimum of a 5% solution of bleach and water) for at least 30 minutes at room temperature
- Rinsing the equipment with reagent water and placing the equipment in an area free of potential *Cryptosporidium* contamination until dry

6.4.2.3 Filter Clogs and Highly Turbid Water Samples

PWSs with highly turbid source waters are likely to generate larger packed pellet volumes after centrifugation and to clog filters than PWSs with low-turbidity waters. As noted in Section 6.1, at least 2 mL of packed pellet volume must be analyzed (for samples in which 10 L is filtered), or as much volume

as two filters can accommodate before clogging. (If more than 10 L is filtered, then less of the packed pellet volume needs to be analyzed.)

PWSs with highly turbid water may be able to collect the required minimum packed pellet volume by avoiding filtration altogether, and shipping a bulk water sample to the laboratory for centrifuging. The laboratory can centrifuge 250-mL or greater aliquot volumes sequentially, until a packed pellet volume of 2 mL is generated.

6.4.3 *E. coli* Sample Collection

For most large systems, *E. coli* analyses will be conducted on-site, so samples will not be shipped in most cases, unlike *Cryptosporidium* samples. However, many small systems will collect *E. coli* samples and ship them off-site for analysis. Regardless of whether the samples are analyzed by the utility's own laboratory or by a commercial laboratory, laboratories analyzing *E. coli* samples for the LT2 rule must use an *E. coli* method approved for use under the rule and must be certified under the drinking water certification program for the general coliform analysis technique corresponding to the method the laboratory plans to use for LT2 rule monitoring [40 CFR part 141.705 (b) and 141.706 (b)]. Approved *E. coli* methods and their corresponding drinking water certification program coliform techniques are discussed in the *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*. Summary information on these methods is also provided in Section 4 of this document.

Collect *E. coli* samples in sterile, non-toxic, plastic, or glass containers with a leak-proof lid. The capacity of sample containers should be 120-mL (6 oz.) or 250-mL (8 oz.) to allow for sufficient sample volume and at least a 1-inch head space to facilitate mixing of the sample by shaking prior to analysis A detailed protocol for collecting source water samples for *E. coli* analysis, as well as packing and shipping guidance for utilities that transport samples off-site for analysis, is provided as **Appendix G**.

EPA strongly encourages laboratories to analyze samples as soon as possible after collection. *E. coli* samples must be analyzed within 24 hours of sample collection [40 CFR part 141.705 (b)(1)]. *Note:* This is a longer time period than currently permitted in *Standard Methods* and the Manual for the Certification of Laboratories Analyzing Drinking Water, and is based on data demonstrating that surface water samples could be held, chilled, for up to 24 hours and still yield valid results (Reference 9.5).

Samples should be maintained above freezing and below 10°C in a refrigerator or in a cooler with wet ice, blue ice, or gel packs, etc. Additional guidance on monitoring sample temperature is available in Section 6.4.5 of this manual.

6.4.4 Measuring Turbidity

PWSs must measure the turbidity of the source at the time of *Cryptosporidium* and *E. coli* sample collection during LT2 rule monitoring. Turbidity must be measured by a party approved by the State [40 CFR part 141.706 (c)] using methods for turbidity measurement approved at 40 CFR part 141.74 [40CFR part 141.705 (c)]. These methods include:

- Method 2130B, published in *Standard Methods for the Examination of Water and Wastewater* (19th or 20th Edition). The full text of the 19th Edition is provided as **Appendix H**.
- Great Lakes Instrument (GLI) Method 2. The full text of this method is provided as **Appendix I**.
- Revised EPA Method 180.1, approved in August 1993 in Methods for the Determination of Inorganic Substances in Environmental Samples (EPA-600/R-93-100). The full text of this method is provided as **Appendix J**.

Systems must use turbidimeters that conform to one of the approved methods for measuring turbidity, such as Hach Turbidimeter 1720D with EPA Method 180.1, GLI Turbidimeter Accu 4 with GLI Method 2, or equivalents (Note: These examples do not constitute an endorsement of specific instrumentation. Approved methods provide specifications that turbidimeters must meet, and conformance of instruments with these particular specifications must be determined prior to analysis.). For regulatory reporting purposes, either an on-line or a benchtop turbidimeter may be used, and systems must comply with all quality control requirements specified in methods and regulations. If a system chooses to utilize on-line units for monitoring, the system must validate the continuous measurements for accuracy on a regular basis using a protocol approved by the State [40 CFR part 141.74 (c) (1)].

6.4.4.1 Measuring Sample Turbidity During LT2 Monitoring

When measuring turbidity, cuvettes must be clear, colorless glass or plastic. The tube must be kept clean, both inside and out, to provide accurate readings. If a sample tube is scratched, it must be discarded.

- **Measuring Sample Turbidity Using SM 2130B.** Measure turbidity immediately after sample collection to prevent temperature changes, particle flocculation, and sedimentation from changing sample characteristics. Shake sample well before pouring into cuvette. Gently agitate to remove air bubbles from the inside of the sample before pouring the sample into cell. Wait until all the air bubbles disappear and remove all moisture from the outside of the sample cell before placing it into the instrument. If fogging occurs, warm the sample by warm water bath for a short time, then re-agitate the sample before placing it in the turbidimeter. Read turbidity directly from instrument display. *Note: Measurements should be within the calibration range.*

- **Measuring Sample Turbidity Using GLI Method 2 or Revised EPA Method 180.1.** Different procedures should be followed, depending on the turbidity of the sample:

  - For turbidities estimated to be less than 40 NTU. Shake the sample thoroughly to disperse the solids. After waiting for the air bubbles to disappear, pour the sample into the turbidimeter tube and read directly from the instrument scale.

  - For turbidities estimated to be greater than 40 NTU. Dilute the sample with turbidity-free water and compute the turbidity with the dilution factor included.

6.4.4.2 General Quality Control for Turbidity Measurements

Utilities performing environmental sample measurements must be approved by the State (or EPA Region, for states that do not have primacy) under the drinking water laboratory certification program [40 CFR part 141.706 (c)]. Each utility laboratory is required to operate a formal quality control (QC) program and to maintain performance records that define the quality of the data generated. Two types of calibration are required for turbidity measurements:

- **A primary suspension standard.** The primary suspension standard should be used to calibrate the turbidimeter initially and at least every four months in order to prevent instrument drift. The calibration should be documented. The standards should be replaced when they exceed the expiration date.

  Acceptable primary suspensions include Formazin (a recipe for preparation can be found at EPA Method 180.1 and Standard Method 2130B), AMCO-AEPA-1 (available from Advanced Polymer Systems), and Hach StablCal Stabilized Formazin Standards (available from Hach Company). Please note that Formazin standards are relatively unstable, particularly at low concentrations. Therefore, dilutions used for calibration need to be prepared on the day they will be used. Stock solutions may be stable for a month (at 400 NTU) to 1 year (at 4000 NTU). Consult an approved method for more information.

- **A secondary suspension standard.** The secondary suspension standard is used daily to check the calibration of the instrument. The calibration should be documented, and should not vary by more than 10% from the initial calibration values (if they do vary by more than 10%, the system should be corrected so that performance is acceptable). The standards should be replaced when they exceed the expiration date.

  Acceptable secondary standards include all primary standards, or other materials that are suggested by instrument manufacturers – such as sealed sample cells filled with a labeled suspension or metal oxide particulates in a polymer gel, or a turbid glass cube. The purpose of the secondary standard is to provide a quick check of calibration. The secondary standards should have a fixed turbidity that does not vary from use to use.

6.4.5 Monitoring Sample Temperature

Source water samples are dynamic environments and, depending on sample constituents and environmental conditions, *Cryptosporidium* oocysts present in a sample can degrade and *E. coli* present in a sample can grow or die off, biasing analytical results. *Cryptosporidium* and *E. coli* samples for LT2 rule monitoring that are not analyzed the same day they are collected must be maintained below 10°C to reduce biological activity. This is specified in Section 8.0 of the June 2003 versions of EPA Method 1622/1623 for *Cryptosporidium* samples and at 40 CFR part 705 (b) (1) and Chapter V, Section 6.3, of the Laboratory Certification Manual (Reference 5.2) for *E. coli* samples.

Samples for all analyses should remain above freezing at all times. This is a requirement in Section 8.0 of the June 2003 versions of EPA Method 1622/1623. Although not a significant concern for 10-L water samples, this is a real concern for *Cryptosporidium* filters and 120- or 250-mL *E. coli* samples that are shipped off-site with coolant materials, such as wet ice, blue ice, or gel packs. *E. coli* holding time studies performed in support of the LT2 rule (Reference 9.5) demonstrated that *E. coli* samples can freeze under these conditions if samples are not packed properly.

The sample collection protocols procedures in **Appendices D, E, F,** and **G** provide sample packing procedures for *E. coli* and *Cryptosporidium* samples. Utility personnel should follow these procedures to ensure that samples remain at acceptable temperatures during shipment.

Because *Cryptosporidium* samples collected for the LT2 rule must meet the QC criteria in the methods [40 CFR part 705 (a) (3)], and because these QC criteria include receipt of samples at <10°C and not frozen, laboratories must reject LT2 *Cryptosporidium* samples received at >10°C or frozen (this is discussed further in Section 3.3.12 in this manual). In these cases, the PWS must re-collect and re-ship the sample.

LT2 rule requirement: *Each sample must meet the QC criteria for the methods [40 CFR part 141.705 (a) (3)]. Per EPA Method 1622/1623, samples not processed on the day of collection must be received at the laboratory at < 10°C and not frozen (Section 8.1)*

The sample collection protocols discussed in Section 6.4.2 for *Cryptosporidium* samples and Section 6.4.3 for *E. coli* samples provide guidance on packing samples to maintain appropriate temperatures. Utility personnel should follow these procedures to ensure that samples remain at acceptable temperatures during shipment.

Several options are available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment:

- **Temperature sample.** One option, for *Cryptosporidium* filtered samples (not for 10-L bulk samples) and *E. coli* 120- and 250-mL samples, is for the PWS to fill a small, inexpensive sample bottle with water and pack this "temperature sample" next to the field sample. The temperature of this extra sample volume is measured upon receipt to estimate the temperature of the field sample. Temperature sample bottles are not appropriate for use with bulk samples because of the potential effect that the difference in sample volume may have in temperature equilibration in the sample cooler. Example product: Cole Parmer cat. no. U-06252-20.

- **Thermometer vial.** A similar option is to use a thermometer that is securely housed in a liquid-filled vial. Unlike temperature samples, the laboratory does not need to perform an additional step to monitor the temperature of the vial upon receipt, but instead just reads the thermometer. Example product: Eagle-Picher Sentry Temperature Vial 3TR-40CS-F or 3TR-40CS.

- **iButton.** Another option for measuring the sample temperature during shipment and upon receipt is a Thermocron® iButton. An iButton is a small, waterproof device that contains a computer chip to record temperature at different time intervals. The information is then downloaded from the iButton onto a computer. The iButton should be placed in a temperature sample in the cooler, rather than placed directly in the cooler, where it may be affected by close contact with the coolant. Information on Thermocron® iButtons is available from http://www.ibutton.com/. Distributors include http://www.pointsix.com/, http://www.rdsdistributing.com, and http://www.scigiene.com/.

- **Stick-on temperature strips.** Another option is for the laboratory to apply a stick-on temperature strip to the outside of the sample container upon receipt at the laboratory. This option does not measure temperature as precisely as the other options, but still mitigates the risk of sample contamination while providing an indication of sample temperature to verify that the sample temperature is acceptable. Example product: Cole Parmer cat. no. U-90316-00.

All temperature measurement devices should be calibrated routinely to ensure accurate measurements. See the U.S. EPA Manual for the Certification of Laboratories Analyzing Drinking Water (Reference 9.3) for more information.

SECTION 7: REVIEWING *CRYPTOSPORIDIUM* DATA

When *Cryptosporidium* samples are processed and analyzed by the laboratory, data on sample measurements, sample processing times, and slide examination results are recorded at the laboratory and reported to the PWS through the LT2 Data Collection System and via hardcopy forms. This section provides an overview of the data recording and reporting processes and discusses the significance of the examination results reported by the laboratory. This section also provides guidance to those PWSs interested in reviewing laboratory data.

7.1 *Cryptosporidium* Data Recording at the Laboratory

The *Cryptosporidium* laboratory records LT2 rule monitoring data using the following standardized forms:

7.1.1 LT2 Sample Collection Form

This form (an example of which is provided as **Appendix C**) is initiated at the plant upon sample collection and is completed at the laboratory. The following information is recorded on this form by the *Cryptosporidium* laboratory:

- Date and time of sample receipt
- Laboratory personnel receiving the sample
- Sample temperature upon receipt
- Sample condition upon receipt

Although none of this information is entered into the LT2 data collection system, it provides documentation for the utility, the laboratory, and EPA or State officials on sample receipt information relevant to LT2 rule requirements regarding sample temperatures and sample holding times.

7.1.2 Method 1622/1623 Bench Sheet

The laboratory uses the bench sheet to record all information associated with sample processing, up to, but not including, sample examination. Information on filtration (if performed in the laboratory), elution, concentration, immunomagnetic separation, and sample staining are documented on this form. These data include:

- Sample ID
- Dates and times for all steps associated with method-required holding times
- All primary measurements used to calculate sample volume analyzed, if less than 100% of the volume filtered was analyzed. This information includes the following:
  - The volume of the sample after the concentrate (packed pellet) has been resuspended
  - The volume of this resuspended concentrate that was actually analyzed

  (These two values are used to calculate the percent of the sample volume analyzed, if less than 100% of the volume filtered was analyzed.)

- Filter clog and packed pellet information, which may need to be provided to demonstrate compliance with LT2 rule sample analysis requirements if less than 10 L was analyzed
- *Cryptosporidium* spiking information for OPR and MS samples
- Analyst names or initials for each step
- Reagent and filter lot information

7.1.3 Method 1622/1623 *Cryptosporidium* Slide Examination Form

The laboratory uses the slide examination form to document detailed information on slide examination. This information includes the following:

- Sample ID
- Date and time the examination was completed
- Positive and negative staining control results
- Detailed information on the characteristics of each object on the slide that the analyst determined was a *Cryptosporidium* oocyst, including the following:
  - Size of the oocyst
  - Shape of the oocyst
  - Whether the DAPI stain applied to the sample revealed the presence of nuclei, and, if so, how many were observed by the analyst
  - Whether the analyst observed internal structures during DIC examination

7.2 Submitting *Cryptosporidium* Data through the LT2 Data Collection System

During the LT2 rule, laboratories will report *Cryptosporidium* data to their PWS clients electronically through EPA's LT2 Data Collection System. The LT2 Data Collection System is a web-based application that allows laboratory users to enter or upload data, then electronically "release" the data to the PWS for review, approval, and submission to EPA and the State. Although ownership of the data resides with the PWS throughout this process, the LT2 Data Collection System increases the ease and efficiency of the data entry and transfer process from one party to another by transferring the ability to access the data from the laboratory to the PWS to EPA and the State, and ensuring that data cannot be viewed or changed by unauthorized parties. A summary of the data entry, review, and transfer process through the LT2 Data Collection System is provided in **Table 7-1**, below.

Table 7-1. LT2 Data Collection System Data Entry, Review, and Transfer Process

Laboratory actions • Laboratory posts analytical results to the LT2 Data Collection System • LT2 Data Collection System reduces data and checks data for completeness and compliance with LT2 rule requirements • Laboratory Principal Analyst confirms that data meet quality control requirements • Laboratory "releases" results electronically to the PWS for review • Laboratory user cannot edit data after it is released to the PWS	EPA does not have access to data
⇩	
PWS actions • PWS cannot edit data - only review data and either return to laboratory to resolve errors or submit to EPA • PWS reviews electronic data through LT2 Data Collection System • PWS "releases" data back to the laboratory if questions • If no questions, PWS submits data to EPA as "approved" or "contested" (indicating that samples have been correctly analyzed, but that the PWS contends are not valid for use in LT2 binning)	
⇩	
EPA and State actions • EPA and State users cannot edit data - only review data • EPA and State review data through LT2 Data Collection System	

The data reporting process is discussed in more detail below, in Sections 7.2.1 through 7.2.3, and discussed in detail in the *Users' Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) Data Collection System*. The LT2 data system users' guide also provides detailed information on the PWS user registration process. Information on the LT2 Data Collection System, as well as a downloadable users' manual, is available at http://www.epa.gov/safewater/lt2/index.html.

7.2.1 Data Entry/Upload

The analyst or another laboratory staff member enters a subset of the data recorded at the bench (Section 7.1) into the LT2 Data Collection System, either by entering the data using web forms or by uploading data in XML format. This information includes the following:

- PWS ID
- Facility ID
- Sample collection point
- Sample collection date
- Sample type (field or MS)
- Sample volume filtered (L), to nearest ¼ L
- Was 100% of filtered volume examined?
- Number of oocysts counted
- For samples in which less than 10 L is filtered or less than 100% of the sample volume is examined, the laboratory also must enter or upload the number of filters used and the packed pellet volume.
- For samples in which less than 100% of sample volume is examined, the laboratory also must report the volume of resuspended concentrate and volume of this resuspension processed through immunomagnetic separation.

- For matrix spike samples, the laboratory also must report the sample volume spiked and estimated number of oocysts. These data are not required for field samples.

The laboratory must verify that all holding times and other QC requirements were met.

After the information has been entered or uploaded into the system, the system will reduce the data to yield final sample results, in oocysts/L, verify that LT2 rule *Cryptosporidium* sample volume analysis requirements were met for samples in which less than 10 L were analyzed (see Section 6.1), and calculate MS recoveries.

The laboratory's Primary Analyst under the Lab QA Program is responsible for verifying the quality and accuracy of all sample results in the laboratory, and is required to review and approve the results before they are submitted to the PWS for review. If inaccuracies or other problems are identified, the primary analyst discusses the sample information with the analyst or data entry staff and resolves the issues before the data are approved for PWS review.

If no inaccuracies or other issues are identified, the Primary Analyst approves the reported data for "release" to the PWS for review (EPA does not receive the data at this point). When the data are approved, the rights to the data are transferred electronically by the system to the PWS, and the data can no longer be changed by the laboratory.

7.2.2 PWS Data Review

After the laboratory has released *Cryptosporidium* data electronically to the PWS using the LT2 Data Collection System, the PWS will review the results. The PWS user cannot edit the data, but if the PWS has an issue with the sample result, such as if the PWS believes that the sample collection point ID or collection date is incorrect, the PWS can release the results back to the laboratory for issue resolution. In addition to noting the reason in the LT2 Data Collection System for the return of the data to the laboratory, you also should contact the laboratory verbally to discuss the issue.

If the PWS determines that the data are accurate, the PWS releases the results to EPA (and the State, if applicable) as "approved" results. If the PWS determines that the data are accurate, but believes that the data are not valid for LT2 binning purposes, the PWS can release the results to EPA and the State as "contested." Contested samples are those that have been correctly analyzed, but that you contend are not valid for use in LT2 binning, and have submitted to EPA for evaluation.

7.2.3 EPA/State Review

After the PWS has released the results as approved or contested, they are available to EPA and State users to review through the LT2 Data Collection System. EPA and State users cannot edit the data.

7.3 What Do the Sample Examination Results Mean?

As noted in Section 3.1, the laboratory applies two stains to a sample slide, and then examines the sample using three different techniques to determine whether objects that cannot be ruled out as *Cryptosporidium* oocysts are on a sample slide. A description of these stains and techniques–and how each is used to evaluate objects examined by the analyst, is provided below.

7.3.1 Immunofluorescent Assay (IFA)

One of the two stains added to the sample before examination is a fluorescent antibody stain that reacts with *Cryptosporidium*. The antibodies in this stain, which exhibit an intense apple-green fluorescence

when the slide is examined using ultraviolet light, will attach to *Cryptosporidium* oocysts that may be present in the sample. During IFA, the analyst scans the entire well at relatively low magnification (200X) for apple-green fluorescing objects the size and shape of oocysts. If such an object is located, the analyst proceeds to the next step in the examination process. The analyst cannot conclude at this stage that an apple-green fluorescing organism the size and shape of a *Cryptosporidium* oocyst is indeed an oocyst because the object may be another organism that has cross-reacted with the antibody stain. Additional examination procedures are used to determine whether this is the case.

7.3.2 4',6-diamadino-2-phenylindole (DAPI) Examination

The second stain added to the sample before examination is DAPI, a dye that interacts with nucleic acids and stains nuclei that may be present within the oocyst. The DAPI stain fluoresces when the slide is examined using ultraviolet light. During the DAPI examination, the analyst observes the object at medium magnification (400X) to determine whether it contains stained nuclei. *Cryptosporidium* oocysts contain four nuclei.

Although looking for four nuclei during DAPI examination, if the object has less than four nuclei, the analyst cannot rule out the possibility that the organism is a *Cryptosporidium* oocyst. For example, if less than four stained nuclei are observed, the object may actually have four nuclei, but some may not be visible in the plane of focus. Similarly, objects in which no stained nuclei are observed may be organisms other than *Cryptosporidium*, may be dead *Cryptosporidium* oocysts, or may even be live oocysts that are resistant to DAPI staining.

The DAPI examination is one of several tools for the analyst to use to determine whether an object is an oocyst. The analyst cannot conclude whether the object is an oocyst based on this examination alone, nor can the analyst conclude, based on negative results, that the organism is non-infectious. As a result, the analyst must proceed to the next step in the examination process, even if less than four nuclei are observed.

7.3.3 Differential Interference Contrast (DIC) Examination

The third evaluation performed by the analyst is an examination of the object at high magnification (1000X). Using DIC, the analyst looks at the object's external or internal morphological characteristics (this does not require the use of a stain). The analyst looks for characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, crystals, spores, etc.). If atypical structures are not observed, and the object cannot be ruled out as an oocyst based on the results of the IFA and the DAPI examination, the analyst reports this object as a *Cryptosporidium* oocyst.

Based on the DIC examination, the size of the object is determined and compared to the acceptable range for the target organism. If the size and shape of the object is within the acceptable range, the analyst records the size and shape and characterizes the *Cryptosporidium* oocyst in one of three ways: (1) an oocyst with internal structures, i.e., those having recognizable structures consistent with *Cryptosporidium*, (2) an oocyst with amorphous structures, or (3) an empty oocyst. Assignment of these characterizations is dependent on analyst judgement and none of these characterizations is a direct indicator of whether oocysts are viable and infectious.

7.5 Reviewing and Validating Raw *Cryptosporidium* Data (*Optional*)

If your PWS plans to review the raw data generated by the laboratory, you should request from the laboratory the hardcopy data needed to verify the electronic results (see Section 5.1.5). However, this step is *not* required. However, for those PWSs interested in taking this extra step, Sections 7.5.1 through 7.5.3 provide guidance on how to review and validate hardcopy data and verify accuracy.

7.5.1 Data Completeness Check

Upon receipt of the hardcopy sample results for a monitoring sample, verify that the laboratory has submitted the following materials:

- **Sample result summary sheet**, which should include the following:
  - Monitoring sample identification information
  - Monitoring sample result, in oocysts/L
  - Laboratory quality control batch associated with the sample
  - Result for the ongoing precision and recovery (OPR) sample analyzed for this QC batch
  - Result for the method blank sample analyzed for this QC batch
- **LT2 sample collection form** initiated by your utility and completed with sample receipt information by the laboratory
- **Method 1622/1623 Bench Sheet** with raw data associated with the monitoring sample (and MS sample, if applicable)
- **Method 1622/1623 *Cryptosporidium* Slide Examination Form** with raw data for the monitoring sample (and MS sample, if applicable)
- **Laboratory comments.** If the laboratory provided comments on the sample analyses or results that require follow-up, contact the laboratory to discuss, if necessary. Comments may include any applicable data qualifiers. The following is a list of potential data qualifiers:
  - The recovery for the associated ongoing precision and recovery (OPR) sample did not met method requirements
  - Oocysts were detected in the method blank
  - Positive and negative staining controls were not acceptable or not examined
  - Method holding times were not met
  - Sample arrived at the laboratory in unacceptable condition

Any of the above data qualifiers would result in the sample being considered invalid for LT2 use and the laboratory should not report the results for the sample to EPA. The PWS may be required to resample.

If forms are missing, incomplete, or incorrect, contact the laboratory immediately to discuss and request resubmission of the missing forms and/or spreadsheets.

7.5.2 Evaluation of Data Against Method Quality Control Requirements

To verify that the laboratory analyzed your monitoring sample within the analytical controls specified by the method, check the following information:

- **Sample condition upon receipt.** Verify on the completed LT2 sample collection form that your sample was received in acceptable condition (not leaking, etc.), and at a temperature between 0°C and 10°C, and not frozen.
- **Method blank.** Verify that the laboratory analyzed a method blank with the monitoring sample's QC batch and confirm that the method blank did not contain any oocysts.

- **Ongoing precision and recovery sample.** Verify that the laboratory analyzed an OPR sample with the monitoring sample's QC batch and that the OPR sample recovery was between 11% and 100%, as required by EPA Methods 1622 and 1623.

- **Holding times.** Using the sample collection date and time on the LT2 data collection form and the dates and times of the method steps recorded by the laboratory on the Method 1622/1623 bench sheet and report form for the monitoring sample, verify the following:
  - The laboratory began elution no more than 96 hours from sample collection
  - The laboratory performed the elution, concentration, purification, and slide preparation (application of the purified sample to the slide) within 1 working day (the date of the elution step should be the same as the date of the slide preparation step)
  - The laboratory stained the slide within 72 hours of application of the purified sample to the slide (generally, the date next to the slide staining step should be no more than 3 days later than the date next to the slide preparation step)
  - The laboratory examined the slide within 7 days of staining (the examination date should be no more than 7 days from the slide staining date)

- **Positive and negative staining controls.** Based on the information at the top of the Method 1622/1623 *Cryptosporidium* reporting form, verify that the laboratory performed positive and negative staining controls, and that the results of these controls were acceptable.

7.5.3 Calculation Verification

The laboratory does not directly report the final concentration of oocysts/L in the sample to EPA. Instead, they report a series of primary measurements that are used by the LT2 data system to automatically calculate the final concentration. The volume filtered, the total volume of resuspended concentrate, and the volume transferred to IMS are used to determine the volume analyzed. The laboratory also records the total count of oocysts detected, which is divided by the volume analyzed to determine the final concentration of oocysts/L. Although the final results are automatically calculated by the LT2 data collection system using the primary measurements supplied by the laboratory, the plant still may wish to verify them. Guidance on recalculating and verifying final results using primary measurements is provided below.

7.5.3.1 Field Sample Calculations

To calculate the concentration of *Cryptosporidium* in your field sample, reported as oocysts/L, the following information is needed:

- Number of oocysts detected in the sample (recorded as a primary measurement from the examination results form)
- Volume analyzed

Using these two data elements, the final concentration is calculated as:

$$\text{final concentration} = \frac{\text{oocysts detected in the sample}}{\text{volume analyzed (L)}}$$

If 100% of the sample volume filtered is examined, then the volume analyzed equals the volume filtered. This applies whether one filter or more than one filter was used; if more than one filter was used, and all

of the volume filtered through the multiple filters is processed through the remainder of the method, then the volume examined is simply the sum of the volumes filtered through each of the filters used.

If < 100% of the volume filtered was processed through the remainder of the method, then additional calculations are needed to determine the volume analyzed. This is discussed below.

Determining Volume Analyzed when Less than 100% of Sample Was Examined

When <100% of the sample filtered is processed through the remainder of the method and examined (such as when the volume filtered yields > 2 mL of packed pellet volume after centrifugation), then the volume analyzed must be determined using the following equations to determine the percentage of the sample that was examined.

$$\text{percent examined} = \frac{\text{total volume of resuspended concentrate transferred to IMS (see Section 7.1.2)}}{\text{total volume of resuspended concentrate produced}}$$

$$\text{volume analyzed (L)} = \text{percent examined} \times \text{sample volume filtered}$$

Determining the Volume of Resuspended Concentrate to Use for Packed Pellets > 5 mL

Packed pellets with a volume >0.5 mL must be divided into subsamples. Use the formula below to determine the total volume of resuspension required in the centrifuge tube before separating the concentrate into two or more subsamples and transferring to IMS.

$$\text{total volume of resuspended concentrate (mL) required} = \frac{\text{pellet volume (mL) after centrifugation}}{0.5\ \text{mL}} \times 5\ \text{mL}$$

Example. A 10-L field sample was filtered and processed, producing a packed pellet volume of 2.7 mL. The laboratory transferred 20 mL of the total resuspended concentrate to IMS and examination. The laboratory detected 20 oocysts during examination. The following calculations were performed to determine the volume analyzed and final concentration.

$$\text{total volume of resuspended concentrate (mL) required} = \frac{2.7\ \text{mL}}{0.5\ \text{mL}} \times 5\ \text{mL} = 27\ \text{mL}$$

$$\text{percent examined} = \frac{20\ \text{mL}}{27\ \text{mL}} = 0.74\ (74\%)$$

$$\text{volume analyzed (L)} = 0.74 \times 10\ \text{L} = 7.4\ \text{L}$$

$$\text{final concentration (oocysts/L)} = \frac{20\ \text{oocysts}}{7.4\ \text{L}} = 2.7\ \text{oocysts/L}$$

7.5.3.2 Matrix Spike Sample Calculations

For matrix spike (MS) samples, the laboratory records all of the same information that is recorded for field samples, in addition to information specific to matrix spike samples. The sample volume spiked and estimated number of oocysts spiked into the sample are used to calculate the concentration of spiked

organisms in the sample. To correct for background concentration, the number of organisms detected in the unspiked field sample is subtracted from the number of oocysts detected in the MS sample.

To determine the percent recovery for a matrix spike (MS) sample, the following information is needed:

- The number of oocysts detected in the MS sample
- The true value of the oocysts spiked into the MS sample
- The number of oocysts detected in the unspiked field sample (to correct for background concentration)

$$\text{percent recovery} = \frac{\text{oocysts counted in MS sample - oocysts counted in unspiked field sample}}{\text{oocysts spiked into MS sample}} \times 100\%$$

7.6 Data Archiving Requirements

LT2 rule monitoring data must keep monitoring results until 36 months after source water monitoring has been completed. Although it is the PWS's responsibility to meet LT2 rule data storage requirements for compliance monitoring samples, the PWS may designate this responsibility to the laboratory.

Although not required, laboratories also can archive slides and/or take photographs of slides to maintain for clients. As noted in Section 5.1.2.5, this may be considered an extra service and result in extra costs, as these steps may not be routinely performed by the laboratory. Slides should be stored in a humid chamber in the dark at 0°C to 10°C. An alternative mounting medium also may be used, which may potentially preserve slides longer. Details are provided in the *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule).*

SECTION 8: REVIEWING *E. COLI* DATA

When *E. coli* samples are processed and analyzed by the laboratory, data on sample measurements, sample processing times, and slide examination results are recorded at the laboratory and reported to the PWS through the LT2 Data Collection System. This section provides an overview of the data recording and reporting processes and provides guidance on how to review the data you receive from the laboratory.

8.1 *E. coli* Laboratory Data Recording at the Laboratory

The laboratories performing *E. coli* analyses during the LT2 rule record the following general types of information:

- Sample identification information
- All primary measurements used to calculate the final *E. coli* concentration for each sample
- The incubation start and read times for each method to verify that method requirements were met
- The name of the analyst performing the sample analysis
- Quality control (QC) analysis results (e.g., positive/negative controls, blanks, etc.)

8.1.1 Sample Identification Information

Sample identification information is used to track the sample through sample collection, analysis, and data reporting. At a minimum, the laboratory records the sample ID, the target parameter (*E. coli*), and the method being used (e.g., Membrane Filtration: SM 9222D/SM 9222G).

8.1.2 Primary Data

The laboratory records all primary measurements needed to calculate the final concentration of E. coli per 100 mL. Primary measurements for membrane filtration methods will include the volumes filtered and the plate counts for each volume filtered. The multiple-well and multiple-tube formats will include the volumes or dilutions of samples analyzed and the number of positive wells or tubes per each volume analyzed.

8.1.3 Sample Processing and Quality Control Information

The laboratory records information on the bench sheet on how they processed and analyzed the sample, including incubation start/end date and times and temperature, and the analyst performing each step of the method. The lot numbers of reagents, media, and materials used to process the sample and the results of QC analyses should be recorded in a media log book or QC checklist. In addition to being used to resolve questions regarding validity of results, this information may be used by the laboratory to determine the source of any problems the laboratory is having with method performance.

8.1.4 Sample Results

The final *E. coli* concentration for field samples will be reported as CFU/100 mL or MPN/100 mL depending on the method used for analysis. If no *E. coli* are detected in the sample, a low censored value based on the volume of sample analyzed must be reported (e.g. <1CFU /100 mL or <1.8 MPN/100 mL). *E. coli* concentration will never be reported as a zero.

8.2 Submission of *E. coli* Data through the LT2 Data Collection System

During the LT2 rule, laboratories will report *E. coli* data electronically through EPA's LT2 Data Collection System to the PWS staff responsible for approving and submitting monitoring results to EPA. The LT2 Data Collection System is a web-based application that allows laboratory users to enter or upload data, then electronically "release" the data to the appropriate PWS staff for review, approval, and submission to EPA and the State. Although ownership of the data resides with the PWS throughout this process, the LT2 Data Collection System increases the ease and efficiency of the data entry and transfer process from one party to another by transferring the ability to access the data from the laboratory to the PWS to EPA and the State, and ensuring that data cannot be viewed or changed by unauthorized parties. A summary of the data entry, review, and transfer process through the LT2 Data Collection System for both *Cryptosporidium* and *E. coli* samples is provided in **Table 7-1**, in Section 7.2, above.

The data reporting process is summarized below, in Sections 8.2.1 through 8.2.3, and discussed in detail in the *Users' Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) Data Collection System*. The LT2 data system users' guide also provides detailed information on the laboratory registration process. Information on the LT2 Data Collection System, as well as a downloadable users' manual, is available at http://www.epa.gov/safewater/lt2/index.html.

8.2.1 Data Entry/Upload

The analyst or another laboratory staff member enters a subset of the data recorded at the bench (Section 8.1) into the LT2 Data Collection System either by entering the data using web forms or by uploading data in XML format. This information includes the following:

- PWS ID
- Facility ID
- Sample collection point
- Sample collection date
- Analytical method number
- Method type
- Source water type (provided by PWS on sample collection form)
- Turbidity result (provided by PWS on sample collection form)
- *E. coli*/100 mL (see note below)

Note: The laboratory may then enter the final result for the sample that was calculated at the laboratory or may enter the primary measurements recorded at the bench, and have the LT2 Data Collection System automatically calculate the final sample concentration. Because this information is specific to method type (membrane filtration, multiple tube fermentation, 51-well, and 97-well), the system provides

different entry screens for each method type. The laboratory staff entering the data should verify that all holding times and other QC specifications were met.

The laboratory's official contact is responsible for verifying the quality and accuracy of all sample results in the laboratory, and is required to review and approve the results before they are submitted to the PWS for review. If inaccuracies or other problems are identified, the official contact discusses the sample information with the analyst or data entry staff and resolves the issues before the data are approved for PWS review.

If no inaccuracies or other issues are identified, the laboratory's official contact approves the data for "release" to the PWS for review (EPA does not receive the data at this point). When the data are approved, the rights to the data are transferred electronically by the system to the PWS, and the data can no longer be changed by the laboratory.

8.2.2 PWS Data Review

After the laboratory has released *E. coli* data electronically to the PWS using the LT2 Data Collection System, the PWS will review the results. The PWS user cannot edit the data, but if the PWS has an issue with the sample result, such as if the PWS believes that the sample collection point ID or collection date is incorrect, the PWS can release the results back to the laboratory for issue resolution. In addition to noting the reason in the LT2 Data Collection System for the return of the data to the laboratory, you also should contact the laboratory verbally to discuss the issue.

If the PWS determines that the data are accurate, the PWS releases the results to EPA (and the State, if applicable) as "approved" results. If the PWS determines that the data are accurate, but believes that the data are not valid for other reasons, the PWS can release the results as "contested."

8.2.3 EPA/State Review

After the PWS has released the results as approved or contested, they are available to EPA and State users to review through the LT2 Data Collection System. EPA and State users cannot edit the data.

8.3 Reviewing and Validating *E. coli* Data (*Optional*)

If the PWS staff responsible for submitting data to EPA plans to review the raw data generated by the laboratory, the original, hardcopy records (whether generated by an in-house laboratory or a contract laboratory) should be compared to the electronic results. However, this step is *not* required. Sections 8.3.1 through 8.3.3 provides guidance on how to review and validate the hardcopy data and verify accuracy.

8.3.1 Data Completeness Check

Upon receipt of hardcopy sample results for a monitoring sample, verify that the following information is included:

- **Sample result summary sheet,** which should include the following:
  - Monitoring sample identification information
  - Monitoring sample result, in *E. coli*/100 mL
  - Laboratory quality control checklist (or other verification from the laboratory that all QC specifications were met)

- **LT2 sample collection form** initiated at the time of sample collection and completed with sample receipt information by the laboratory

- ***E. coli* Method Bench Sheet** completed by the laboratory with primary sample processing and analysis data associated with the monitoring sample

- **Laboratory comments.** If the laboratory provided comments on the sample analyses or results that require follow-up, contact the laboratory to discuss, if necessary. Comments may include any applicable data qualifiers. The following is a list of potential data qualifiers:
  - Sample arrived at the laboratory in unacceptable condition (i.e., leaking)
  - Sample holding time exceeded
  - Sample holding temperature not within acceptable range
  - Unacceptable blank sample result
  - Unacceptable positive or negative control result
  - Media sterility checks were not acceptable
  - Method incubation times or temperatures were not within acceptable range
  - Membrane filtration: Too much sediment on the filter
  - Membrane filtration: Confluent growth of non-target organism
  - Membrane filtration: Colonies too numerous to count (TNTC)
  - Membrane filtration: Pre- or post- filtration series sterility check not acceptable (e.g., contamination with *E. coli* organism)
  - Quanti-Tray® was damaged or leaked
  - Sample was not distributed to all wells in Quanti-Tray®
  - All rows of tubes were not inoculated
  - Positive presumptive tubes were not transferred into the appropriate confirmatory medium

Any of the above data qualifiers would result in the sample being considered invalid for LT2 use and the results for the sample should not be entered into the LT2 data collection system. If the laboratory enters the results into the LT2 data collection system, the PWS should not submit the results to EPA.

If forms are missing, incomplete, or incorrect, contact the laboratory immediately to discuss and request resubmission of the missing forms and/or spreadsheets.

8.3.2 Evaluation of Data Against Method Quality Control Requirements

To verify that the laboratory analyzed your monitoring sample within the analytical controls specified by the method, check the following information:

- **Sample condition upon receipt.** If the sample was shipped to the laboratory, verify on the completed LT2 sample collection form that your sample was received in acceptable condition (e.g., not leaking, etc.), and at a temperature below 10°C, but not frozen.

- **QC samples associated field samples.** The frequency of analysis of quality control samples including method blanks, positive and negative controls, etc. varies according to method requirements

and specifications in the Certification Manual. Verify that the required QC samples were run with the field sample. A summary of these QC specifications is provided in Section 4.2 of this document.

- **Holding time.** Using the sample collection date and time on the LT2 data collection form and the date and time of the first method step recorded by the laboratory on the *E. coli* method bench sheet, verify that the laboratory began sample analysis within 24 hours of sample collection.

- **Incubation times and temperatures.** Using the dates and times of the method steps recorded by the laboratory on the *E. coli* method bench sheet, verify that the method-specified incubation times and temperatures, specified in **Table 8-1** were met.

Table 8-1. Incubation Times and Temperatures for Approved *E. Coli* Methods

Method	Media	Incubation Time/Temperature
Standard Methods 9223B	Colilert ®	24 to 28 hours at 35°C ± 0.5°C
	Colilert-18 ®	18 to 22 hours at 35°C ± 0.5°C
Standard Methods 9221B/F	LTB	24 ± 2 and 48 ± 3 hours at 35°C ± 0.5°C
	EC-MUG	24 ± 2 hours at 44.5°C ± 0.2°C
Standard Methods 9222B/9222G	mENDO → NA-MUG	24 ± 2 hours at 35°C ± 0.5°C → 4 hours at 35°C ± 0.5°C
	LES-ENDO → NA-MUG	24 ± 2 hours at 35°C ± 0.5°C → 4 hours at 35°C ± 0.5°C
Standard Methods 9222D/9222G	mFC → NA-MUG	24 ± 2 hours at 44.5°C ± 0.2°C → 4 hours at 35°C ± 0.5°C
Standard Methods 9213D	mTEC agar	2 hours at 35°C ± 0.5°C → 22 to 24 hours at 44.5°C ± 0.2°C
EPA 1603	Modified mTEC	2 hours at 35°C ± 0.5°C → 22 to 24 hours at 44.5°C ± 0.2°C
EPA 1604	MI medium	24 hours at 35°C ± 0.5°C
Other Membrane Filter Method	m-ColiBlue24®® Broth	24 hours at 35°C ± 0.5°C

8.3.3 Calculation Verification

Method-specific data to record for each of the individual method types as well as standardized calculations for each method type are discussed in Sections 8.3.3.1 through 8.3.3.4.

8.3.3.1 Calculations for Determining the *E. coli* Concentration Using the Colilert® Quanti-Tray 2000® (97-well)

A. **Select appropriate dilution to yield countable results.** If multiple dilutions are used, the tray exhibiting positive wells in the 40% to 80% range (39 to 78 total positive large and small wells) should be used to determine MPN value.

B. **Determine MPN.** Using the number of large positive wells and small positive wells from the appropriate dilution, identify the corresponding MPN/100 mL in the table provided by the vendor. Large well values are located in the left column; small well values are located in the top row. For example, if a 100-mL sample was analyzed, and there were 29 large positive wells and 5 small positive wells, the corresponding MPN would be 49.6 MPN/100 mL.

C. **Adjust for dilution factor.** Because the MPN/100 mL values in the table are based on 100-mL samples, the MPN value should be adjusted if less than 100-mL of sample volume was analyzed. Use the following calculation to adjust the MPN to account for the dilution:

$$\text{Analytical result} = \frac{\text{MPN value}}{\text{mL of sample analyzed}}$$

Example:

Volume analyzed = 10 mL of sample (in 90 mL of dilution water)
Large wells positive = 39
Small wells positive = 5
The MPN value calculated based on the intersection of 10 and 2 in the table.
MPN = 81.3

$$\text{Analytical result} = 81.3 \times \frac{100}{10} = 813 \textit{ E. coli} \text{ MPN/100 mL}$$

8.3.3.2 Calculations for Determining the *E. coli* Concentration Using the Colilert® Quanti-Tray 51® (51-well)

A. **Select appropriate dilution.** If multiple dilutions are used, the tray exhibiting positive wells around the 80% range (41 positive wells) should be used to determine MPN value.

B. **Determine MPN.** Using the number of positive wells from the appropriate dilution, identify the corresponding MPN/100 mL in the table provided by the vendor. For example, if a 100-mL sample was analyzed, and there were 26 positive wells, the corresponding MPN would be 36.4 MPN/100 mL

C. **Adjust for dilution factor.** Because the MPN/100 mL values in the table are based on 100-mL samples, the MPN value should be adjusted if less than 100-mL of sample volume was analyzed. Use the following calculation to adjust the MPN to account for the dilution:

$$\text{MPN value} \times \frac{100}{\text{mL sample analyzed}} = \textit{E. coli} \text{ MPN/100 mL}$$

Example:

Volume analyzed (mL) = 10 mL (in 90 mL of dilution water)
Number of positive wells = 41
MPN = 83.1

The analytical result is calculated as follows:

$$83.1 \times \frac{100}{10} = 831 \textit{ E. coli} \text{ MPN/100 mL}$$

8.3.3.3 Calculations for determining the *E. coli* concentration using membrane filter data (adapted from Reference 9.4)

A. *E. coli* counts should be determined from the volume(s) filtered that yielded 20 to 80 *E. coli* colonies (20-60 for mFC-NA-MUG), and not more than 200 total colonies per plate. (Guidance for samples that do not yield countable plates is provided in Sections E and F)

Note: The analytical result can be automatically calculated using the LT2 Data Collection System. See Section 8.2.1 for additional information.

B. If there are greater than 200 colonies per membrane, even for the lowest dilution, the result is recorded as "too numerous to count" (TNTC). These results cannot be reported for LT2 monitoring, as they cannot be used for the required data analyses. During the next sampling event, analyze an additional, lower dilution volume (the highest dilution volume may be omitted) unless conditions were unusual (e.g., heavy rains, flooding, etc.) during the sampling event yielding TNTC for all dilutions.

C. If colonies are not sufficiently distinct for accurate counting, the result is recorded as "confluent growth" (CNFG). To prevent CNFG from occurring, smaller sample aliquots should be filtered. For example, if sample volumes of 100, 10, 1 and 0.1 mL are analyzed and even the 0.1-mL plates results in CNFG, then potentially 0.01 mL should be analyzed during the next sampling event. The 100-mL volume can be eliminated. *Note: If growth is due to high levels of total coliforms but low E. coli then another method should be chosen for analyses that does not rely on total coliform determination prior to or simultaneously with E. coli determination.*

Note: *Results that are TNTC or CNFG are not appropriate for LT2 microbial data analysis, and cannot be entered into the LT2 Data Collection System.*

D. Using the *E. coli* counts from the appropriate dilution, *E. coli* CFU/100 mL is calculated based on the following equation:

$$\textit{E. coli}\ \text{CFU} \times \frac{100}{\text{mL sample filtered}} = \textit{E. coli}\ \text{CFU/100 mL}$$

Example 1:

Filter 1 volume = 100 mL	CFU = TNTC
Filter 2 volume = 10 mL	CFU = 40
Filter 3 volume = 1.0 mL	CFU = 9
Filter 4 volume = 0.1 mL	CFU = 0

Using the guidance on countable colonies in Step A, the counts from the 10-mL plate will be used to calculate the *E. coli* concentration for the sample:

$$40\ \textit{E. coli}\ \text{CFU} \times \frac{100}{10\ \text{mL}} = 400\ \textit{E. coli}\ \text{CFU/100 mL}$$

E. If no *E. coli* colonies are present, the detection limit is calculated as < largest volume filtered per 100 mL.

Example 2:

Filter 1 volume (mL) = 100 mL CFU = 0
Filter 2 volume (mL) = 10 mL CFU = 0
Filter 3 volume (mL) = 1.0 mL CFU = 0

$$\text{Detection limit} = \frac{100\ \text{mL}}{\text{Largest volume filtered}} = \textit{E. coli}\ \text{CFU/100 mL}$$

$$\frac{100\ \text{mL}}{100\ \text{mL}} = <1\ \textit{E. coli}\ /100\ \text{mL}$$

Example 3:

Filter 1 volume (mL) = 100 mL CFU = Lab accident, no data available
Filter 2 volume (mL) = 10 mL CFU = 0
Filter 3 volume (mL) = 1.0 mL CFU = 0

Calculation of *E. coli*/100 mL:

$$\frac{100\ \text{mL}}{10\ \text{mL}} = <10\ \textit{E. coli}\ \text{CFU}\ /100\ \text{mL}$$

F. If there are no filters with *E. coli* counts in the 20-80 colony range (20-60 for mFC-NA-MUG), sum the *E. coli* counts on all filters, divide by the volume filtered and report as number per 100 mL.

Example 4:

Filter 1 volume (mL) = 50 mL CFU = 15
Filter 2 volume (mL) = 25 mL CFU = 6
Filter 3 volume (mL) = 10 mL CFU = 0

The analytical result is calculated as:

$$(15 + 6 + 0) \times \frac{100}{(50+25+10)} = 25\ \textit{E. coli}\ \text{CFU/100 mL}$$

Example 5:

Filter 1 volume (mL) = 50 mL CFU = 105
Filter 2 volume (mL) = 25 mL CFU = 92
Filter 3 volume (mL) = 10 mL CFU = 85

The analytical result is calculated as:

$$(105 + 92 + 85) \times \frac{100}{(50 + 25 + 10)} = 332\ \textit{E. coli}\ \text{CFU/100 mL}$$

Example 6:

Filter 1 volume (mL) = 100 mL	CFU = 82
Filter 2 volume (mL) = 10 mL	CFU = 18
Filter 3 volume (mL) = 1.0 mL	CFU = 0

The analytical result is calculated as:

$$(82 + 18 + 0) \times \frac{100}{(100 + 10 + 1)} = 90 \textit{ E. coli} \text{ CFU/100 mL}$$

Example 7:

Filter 1 volume (mL) = 50 mL	CFU = TNTC
Filter 2 volume (mL) = 25 mL	CFU = TNTC
Filter 3 volume (mL) = 10 mL	CFU = 83

The analytical result is calculated as:

$$83 \times \frac{100}{10} = 830 \textit{ E. coli} \text{ CFU/100 mL}$$

8.3.3.4 Calculation of *E. coli* Concentrations Using Multiple-Tube Methods (adapted from Reference 9.6):

The guidance and examples for determining *E. coli* concentrations using multiple-tube methods are based on the revision of Standard Methods 9221C included in the *2001 Supplement to the 20th Edition of Standard Methods*, approved by the Standard Methods Committee in 1999.

Note: The analytical result can be automatically calculated using the LT2 Data Collection System. See Section 8.2.1 for additional information.

A. For each sample volume (e.g., 10, 1, 0.1, and 0.01 mL or additional sample volumes as necessary), determine the number of positive tubes out of five.

B. A dilution refers to the volume of original sample that was inoculated into each series of tubes. Only three of the dilution series will be used to estimate the MPN. The three selected dilutions are called significant dilutions and are selected according to the following criteria. Examples of significant dilution selections are provided in **Table 8-2**, below.

- Choose the highest dilution (the most dilute, with the least amount of sample) giving positive results in all five tubes inoculated and the two succeeding higher (more dilute) dilutions. (**Table 8-2**, Example A).

- If the lowest dilution (least dilute) tested has less than five tubes with positive results, select it and the two next succeeding higher dilutions (**Table 8-2**, Examples B and C).

- When a positive result occurs in a dilution higher (more dilute) than the three significant dilutions selected according to the rules above, change the selection to the lowest dilution (least dilute) that has less than five positive results and the next two higher dilutions (more dilute) (**Table 8-2**, Example D).

- When the selection rules above have left unselected any higher dilutions (more dilute) with positive results, add those higher-dilution positive results to the results for the highest selected dilution (**Table 8-2**, Example E).

- If there were not enough higher dilutions tested to select three dilutions, then select the next lower dilution (**Table 8-2**, Example F).

C. MPN values need to be adjusted based on the significant dilutions series selected above. Because the MPN/100 mL values in the table are based on the 10 mL, 1 mL, and 0.1 mL dilution series, per method requirements, the MPN value must be adjusted if these are not the significant dilution series selected. Use the following calculation to adjust the MPN when the 10 mL, 1 mL, and 0.1 mL dilution series are not the significant dilution series selected:

$$\text{Analytical result} = \frac{\text{MPN value}}{\text{\# of mL in middle dilution}} = \textit{E. coli}\ \text{MPN/100 mL}$$

Table 8-2. Examples of Different Combinations of Positive Tubes (Significant Dilution Results Are in *Bold* and Underlined)

Example	Least dilute (Lowest) → Most dilute (Highest)					Combination of positives	MPN Index from Standard Methods	*E. coli*/100 mL (after adjustment)
	10 mL	1 mL	0.1 mL	0.01 mL	0.001 mL			
A	5	***5***	***1***	***0***	0	5-1-0	33	330
B	***4***	***5***	***1***	0	0	4-5-1	48	48
C	***0***	***0***	***1***	0	0	0-0-1	1.8	1.8
D	5	***4***	***4***	***1***	0	4-4-1	40	400
E	5	***4***	***4***	***0***	***1***	4-4-1	40	400
F	5	5	***5***	***5***	***2***	5-5-2	540	54,000

Example A: The significant dilution series for the 5-1-0 combination of positives includes the 1 mL, 0.1 mL, and 0.01 mL dilutions. Since the 10 mL, 1 mL, and 0.1 mL dilutions were not selected, an adjustment is necessary to account for the dilutions selected:

$$\text{Analytical result} = \frac{33}{0.1} = 330\ \textit{E. coli}\ /\ 100\ \text{mL}$$

Example B: Since the 10 mL, 1 mL, and 0.1 mL dilutions are the significant dilutions, no adjustment is necessary and the result is 48 *E. coli*/100 mL.

Example C: Since the 10 mL, 1 mL, and 0.1 mL dilutions are the significant dilutions, no adjustment is necessary and the result is 1.8 *E. coli*/100 mL.

Examples D and E: The significant dilution series for the 4-4-1 combination of positives includes the 1 mL, 0.1 mL, and 0.01 mL dilutions. Since the 10 mL, 1 mL, and 0.1 mL dilutions were not selected, an adjustment is necessary to account for the dilutions selected:

$$\text{Analytical result} = \frac{40}{0.1} = 400 \textit{ E. coli} / 100 \text{ mL}$$

Example F: The significant dilution series for the 5-5-2 combination of positives includes the 0.1 mL, 0.01 mL and 0.001 mL dilutions. Since the 10 mL, 1 mL, and 0.1 mL dilutions were not selected, an adjustment is necessary to account for the dilutions selected:

$$\text{Analytical result} = \frac{540}{0.01} = 54{,}000 \textit{ E. coli} / 100 \text{ mL}$$

8.4 Data Archiving Requirements

Under the LT2 rule, monitoring data must keep until 36 months after source water monitoring has been completed [40 CFR part 141.731 (a)]. Although it is the PWS's responsibility to meet LT2 rule data storage requirements for compliance monitoring samples, the PWS may designate this responsibility to the laboratory.

SECTION 9: REFERENCES

9.1 Connell, Kevin, et al. 2000. ICRSS - Building a Better Protozoa Data Set, J. AWWA. 91(10): 30 - 43.

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9.3 USEPA. 1997. Manual for the Certification of Laboratories Analyzing Drinking Water; Criteria and Procedures; Quality Assurance: Fourth Edition. EPA 815-B-97-001.

9.4 APHA. 1998. Standard Methods for the Examination of Water and Wastewater; 20th Edition. American Public Health Association, American Water Works Association, Washington, D.C.

9.5 Pope, M., et al. 2002. Assessment of the effects of holding time and temperature on *E. coli* concentrations in surface water samples. Appl. Environ. Micro. (submitted).

9.6 2001 Supplement to the 20th Edition of Standard Methods 9221 C: Explanation of Bacterial Density. This supplement is available for download at http://www.techstreet.com/cgi-bin/detail?product_id=923645.

SECTION 10: ACRONYMS

CFU	Colony-forming unit
CNFG	Confluent growth
DAPI	4, 6-diamidino-2-phenylindole
DIC	Differential interference contrast
EPA	U.S. Environmental Protection Agency
FA	Immunofluorescense assay
FITC	Fluorescien isothiocyanate
GWUDI	Ground water under the direct influence of surface water
ICR	Information Collection Rule
IFA	Immunofluorescence assay
IMS	Immunomagnetic separation
IPR	Initial precision and recovery
IPT	Initial proficiency testing
L	Liter
LT2 rule	Long Term 2 Enhanced Surface Water Treatment Rule
LT2ESWTR	Long Term 2 Enhanced Surface Water Treatment Rule
MPN	Most probable number
MS	Matrix spike
MS/MSD	Matrix spike/matrix spike duplicate
µm	Micrometer
NA-MUG	Nutrient agar (NA) with 4-methylumbelliferyl-beta-D-glucuronide (MUG)
nm	Nanometer
NPDWR	National Primary Drinking Water Regulations
NTU	Nephelometric turbidity unit
OPR	Ongoing precision and recovery
OPT	Ongoing proficiency testing
PBMS	Performance-based measurement system
PT	Proficiency testing
PWS	Public water system
QA	Quality assurance

QAP	Quality assurance plan
QC	Quality control
RSD	Relative standard deviation
SDWA	Safe Drinking Water Act
TNTC	Too numerous to count
UV	Ultraviolet